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# The Lymphatic System in Health and Disease

J. Winny Yun
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*FROM MOLECULE TO FUNCTION TO DISEASE*

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# The Lymphatic System in Health and Disease

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## ABSTRACT

The lymphatic system develops and functions in parallel with the blood circulatory system (termed the "hemovasculature") and accomplishes transport of interstitial fluids, dietary lipids, and reverse transport of cholesterol, immune cells, and antigens—providing a critical homeostatic fluid balance and transmission of immune cells and mediators back to the cardiovascular system. Although the daily flow of lymph (normally 1–2 L/day under unstressed conditions) is far lower than that of daily blood flow (which is 7,500 L/day), without the adequate functioning of the lymphatics, virtually all organs and tissues would acutely suffer many different physical and inflammatory stresses ranging from edema to organ system failure. Although blood and lymphatic vessels often form in anatomic parallels to one another, our knowledge of the workings of the lymphatic system, the fine structure of lymphatic networks, how they function in different organs, and how they are regulated physiologically and immunologically are far from parallel; our knowledge of the lymphatic system still remains at only a tiny fraction of what is understood about the cardiovascular system. Although both the cardiovascular and lymphatic systems are important transport systems, what they transport and how they transport and propel these very different cargoes could not be more dissimilar.

This book provides an overview of the history of the discovery (and re-discovery) of the components of the lymphatic system, lymphatic anatomy, physiological functions of lymphatics, molecular features of the lymphatic system, and clinical perspectives involving lymphatics which may be of interest to scientists, clinicians, patients, and the lay public. We provide a current understanding of some of the more important structural similarities and differences between lymphatics and the blood vascular system, their coordinated control by angiogenic and hemangiogenic growth factors and other modulators, the fate and lineage determinants which control lymphatic development, and the roles that lymphatics may play in several different diseases.



## KEY WORDS

lymphatics, lymphatic system, lymphedema, lymphatic endothelial cells, lymphangiogenesis, glymphatic, lymphedema distichiasis, Prox-1, FOXC2, VEGFR-3, LYVE-1, VEGF-C, VEGF-D

# Dedication

This book is dedicated to the memory of Dr. Harris Granger, PhD (1944–2018), a leading cardiovascular researcher active in the field for many decades.

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# Preface

The lymphatic system develops and functions in parallel with the blood circulatory system (termed the "hemovasculature") and accomplishes transport of interstitial fluids, dietary lipids, and reverse transport of cholesterol, immune cells, and antigens—providing a critical homeostatic fluid balance and transmission of immune cells and mediators back to the cardiovascular system. Although the daily flow of lymph (normally 1–2 L/day under unstressed conditions) is far lower than that of daily blood flow (which is 7,500 liters/day), without the adequate functioning of the lymphatics, virtually all organs and tissues would acutely suffer many different physical and inflammatory stresses ranging from edema to organ system failure.

Although blood and lymphatic vessels often form in anatomic parallels to one another, our knowledge of the workings of the lymphatic system, the fine structure of lymphatic networks, how they function in different organs, and how they are regulated physiologically and immunologically are far from parallel; our knowledge of the lymphatic system still remains at only a tiny fraction of what is understood about the cardiovascular system. Although both the cardiovascular and lymphatic systems are important transport systems, what they transport and how they transport and propel these very different cargoes could not be more dissimilar. Here, we provide a current understanding of some of the more important structural similarities and differences between lymphatics and the blood vascular system, their coordinated control by angiogenic and hemangiogenic growth factors and other modulators, the fate and lineage determinants which control lymphatic development, and the roles that lymphatics may play in several different diseases.

Because of the explosive research in this area, we have found it difficult to adequately summarize the very many recent findings on lymphatic biology in one book, but we have attempted to provide an overview of the history of the discovery (and re-discovery) of the components of the lymphatic system, lymphatic anatomy, physiological functions of lymphatics, molecular features of the lymphatic system, and clinical perspectives involving lymphatics which may be of interest to scientists, clinicians, patients, and the lay public.

Congenital dysregulation or destruction of lymphatics as a result of disease or due to surgery or radiotherapy can produce primary and secondary forms of lymphedema. These

conditions are debilitating in their own right and also magnify stress and injury in several clinical conditions, which diminish quality of life issues and increase morbidity. Therefore, a greater appreciation of how the lymphatic system can repair itself may also yield valuable new treatments for cancer, inflammation, and improve treatments for the many forms of lymphedema and lymphedema-related conditions.

CHAPTER 1

# Introduction

Our understanding of the organization and regulation of the *blood vascular system* and blood vessel angiogenesis over the past three decades has irreversibly changed how clinicians perceive many diseases and how two main lethal divisions of disease, cancer and cardiovascular disease, are understood, diagnosed, and treated. Investigators have recently witnessed an intense resurgence of research interest on the lymphovascular system and lymphatic biology. Although long-marginalized, studies on lymphatics have the potential to equal or exceed that of blood vascular biology (the terms "blood" and "lymphatic" used to distinguish the two systems.)

Optically, this historical oversight literally reflects the apparent difficulties in visualizing lymphatics as well as their very low flow rate making it difficult to see them or properly appreciate their slower but unrelenting transport functions, which like the blood vascular system reflect flow and propulsion Consequently, until very recently, relatively less effort was expended on developing methods and approaches to study lymphatics. Presently, the field lymphatic biology is witnessing a resurgence of interest in the importance of lymphatics in health and disease, especially among clinicians. These observations and findings are not only valuable with regard to lymphedema treatment and cancer therapy, but the recognition of the important roles played by lymphatics will also aid in the understanding and treatment of acute and chronic forms of inflammation.

Herewith, we provide an overview of the lymphatic system, its development and regulation, its roles in several organ systems, and novel perspectives in applying these findings as diagnostic, prognostic, and therapeutic targets.

## 1.1 LYMPHATICS IN HISTORY

Although blood is red, lymph is clear or white (depending on dietary consumption of fats) anatomical location and filling making them very hard to spot on dissection. Larger lymphatics are often partially collapsed and also hard to see. Therefore, compared to blood filled vessels which are distinctive based on their red (arteries) or blue (veins) color and rapid, pulsing flow,

lymphatics can be almost invisible. The word "lymph" apparently derives from the Greek word for "Nymph" ("νύμφη"), a description of female spirits associated with water [1] and from the Roman deity of pure water "*Lympha*" [2, 3].

Despite the varying visibility of these networks, lymphatics were still noticed by many scientific anatomists over history and were described multiple times even in ancient history with mentions of them in Egyptian, Chinese, and Greek writings and texts. The first macroscopic component of the lymphatic system described were likely lymph nodes, which were probably reported by Hippocrates who described nodes in the neck and groin as well as those surrounding the intestines and kidneys. Hippocrates (460–370 BC) may have first described lymphatics in his treatise *Peri adenôn* [4] in which the word for gland (*adenes*) is used first as well. Hippocrates may also have accurately anticipated roles that lymphatics play in controlling interstitial fluid balance and may also have suggested that lymphatics might participate in inflammation [5]. The lymphatic *vessels* which enter and leave these nodes are by comparison, much more difficult to visualize without assistance. In the small intestine, lymphatic vessels are most easily seen following the consumption of a fat-laden meal, where fats incorporated into chylomicrons give the lymphatics a milky-white appearance following fat absorption, but otherwise these vessels can be very difficult to see, and small vessels remain almost imperceptible to the eye.

Possibly the first recorded description of lymphatic vessels was by the physician Herophilus (335–280 BC) and by the anatomist and royal physician Eristatratus (310–250 BC) in Alexandria, who observed lymphatics but did not designate their function. Later, Ruphus (98–117 AD) and Galen (129–199 AD) dissected pigs and apes and found their lymphatics; Galen may have believed that lymphatics represented a type of blood vessel rather than a distinctive transport system. After a pause of more than a thousand years in lymphatic study, the Italian anatomist Gaspare Asseli (1581–1626) (also known as Asselius), living in Milan, again noted (in 1622) the post-feeding visibility of lymphatics as a function of the time after feeding. He died before his master work on lymphatics, *De lactibus sive lacteis venis* (Of rich milk, or milky veins) (1627), was published, within a year of his death. Therefore, the movement of lymph appears to actually have been described before William Harvey described the flow of blood in his famous 1628 book titled *De Motu Cordis* (or On the Motion of the Heart and Blood). The French scientist Jean Pecquet (1622–1674) is memorialized for having finally linked these two systems when describing the *Pecquet's cistern* (which is now called the Cisterna Chyli), a chamber at the bottom of the thoracic duct in which intestinal and lumbar lymph trunks empty their contents before transferring to the thoracic duct and then to subclavian veins into the circulatory system.

Later, Olaf Rudbeck, a Swedish scientist and author (1630–1678) recognized lymphatics as a system equivalent to that of the blood circulation; he also noted thoracic ductal links between lymphatics and the blood circulation and recorded lymphatic structure in the esopha-

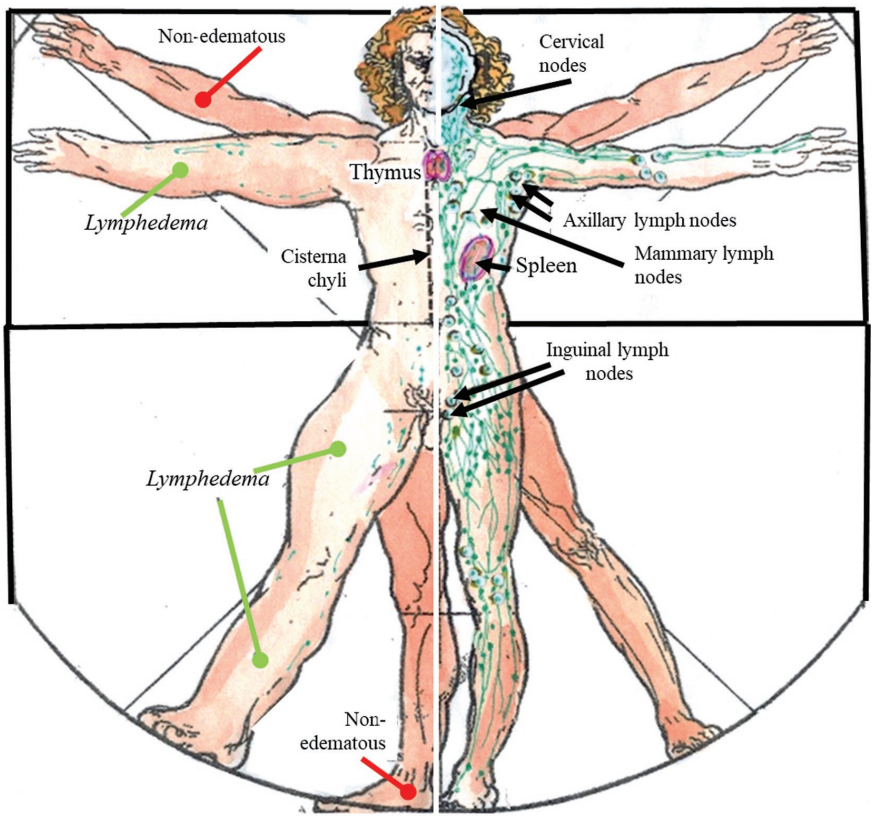


**FIGURE 1:** The lymphatic system. Right side shows the lymphatic nodes and organs in their approximate placement. Left side shows profiles of normal vs. lymphedematous limbs.

gus, colon, and the liver. Importantly, Rudbeck also appears to have first connected lymphatic disturbances with the development of edema (tissue swelling) and ascites (fluid collection in the abdomen) [6]. The Danish mathematician and theologian Thomas Bartholin (1616–1680) published the first full description of the lymphatic system in 1652. After many intervening physical descriptions of the components of the lymphatic system by Niels Stensen (1638–1686) and Giovanni Guglielmo Riva (1627–1677), Carl Friedrich Wilhelm Ludwig (1816–1896) realized that lymph was an ultrafiltrate of plasma, which was finally characterized by Ernest Henry Starling (1866–1927), known for defining the forces that promote capillary exchange of water and solutes. Then, Florence Rena Sabin (1871–1953), working at Johns Hopkins University, produced one of the first descriptions of the embryological development of the lymphatic system, an achievement that continues to guide the field to this day.

Currently, many groups of investigators are examining roles played by lymphatics in immune surveillance, cancer, inflammation, blood pressure regulation, and reverse cholesterol

transport [7]. Hereditary disturbances in lymphatic patterning during development can provoke several forms of #congenital lymphedema, as well as post-natal and adolescent onset forms of lymphedema. Besides genetically induced forms of lymphedema and surgically or radiation induced disturbances in lymphatics that impair remodeling, reorganization or re-establishment of lymphatic communications appear to contribute to secondary forms of lymphedema, especially limb edema. We are now also recognizing how lymphatic disturbances may also contribute to atherosclerosis, tumor metastasis, obesity, and forms of chronic inflammation such as inflammatory bowel diseases. In the following sections, we review lymphatic vascular regulation and compare lymphatic structure and function in different organ systems, with an emphasis on their normal roles in health and how dysfunction contributes to many disease states.

## 1.2 THE LYMPHATIC SYSTEM

Under normal conditions, the human lymphatic system creates around 1–2 liters of lymph [8], and this volume can be increased several times during inflammation; lymph flow does not parallel blood flow. Lymph is a liquid ultrafiltrate of blood plasma that contains proteins, immune cells (including dendritic cells and T cells), and lipids that continually escape the vascular compartment to enter the interstitial spaces, and ultimately return to the blood circulation via the lymphatic system.

Interstitial fluid first forms in tissues as plasma is filtered across the blood vascular endothelium to enter the "interstitium," or the space between cells in tissues. Our understanding of the exact composition of lymph has been limited by several technical issues, most importantly the difficulty in cannulating lymphatics and the very slow rate of lymph formation. Interstitial fluid formed by this process is propelled into blind-ended "initial lymphatic capillaries" by the pressure gradient between interstitium and lymphatic lumen. The initial lymphatic capillary have overlapping "flap-like" valves that facilitate the entry of interstitial fluid into lymphatics, but not reflux (under normal conditions) back into tissues.

Lymphatic endothelial cells are also connected to the tissues (in which fluid is collected) by anchoring filaments that pull these flaps open whenever interstitial fluid pressure is high, an event that greatly facilitates the movement of lymph into these channels. Lymph from initial lymphatics is then conducted into lymphatic collector vessels that maintain unidirectional flow and prevent lymph reflux due to the presence of valves. Although lymphatic capillaries have flap-like button junctions, lymphatic collectors are often encased by white adipose tissue and seem to permit antigens penetration into the lymphatic lumen outside of the initial lymphatics, which is a relatively

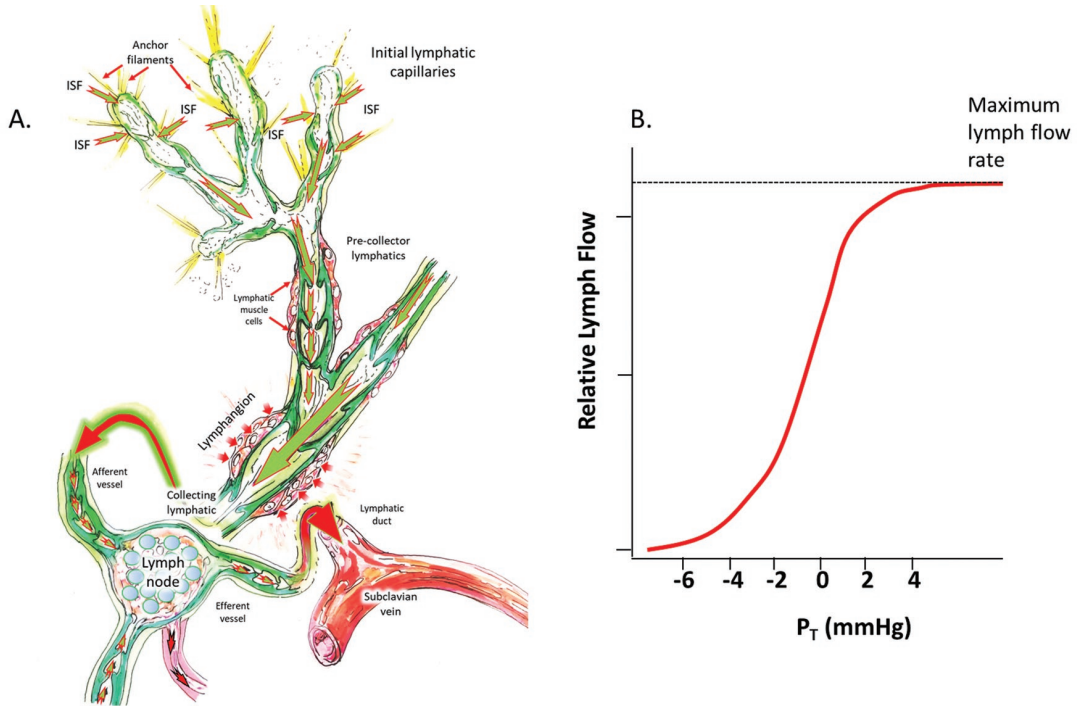


**FIGURE 2:** A) Initial lymphatic and adjacent filtering capillary illustrate the creation of a filtrate, its entry into the lymph microvessels, and its ultimate return to the cardiovascular system at the subclavian veins. B) The graph shows the relationship between lymph flow and increasing interstitial fluid pressures.

new paradigm of lymphatic collection. When the barrier function of the collector lymphatic is diminished, such as in Prox-1 deficiency (a governor of lymphatic specification) [9], lipids may accumulate outside of lymphatic vessels, possibly contributing to fat deposition, and lymphatic disturbances have been suggested as a mechanism for fat accumulation in obesity [10].

Lymph that is formed by normal and pathological conditions enters the lymphatic lumen and is actively propelled by the contractile action of lymphangions that force lymph forward against a hydrostatic fluid pressure gradient [11]. Lymphangions are lymphatic segments which are bound by two unidirectional valves that are compressed by rhythmic tissue compression produced by 'intrinsic' phasic contractions (Figure 3) and tonic contractions of specialized lymphatic smooth muscle cells, which have a distinctive molecular signature and regulation [12]. These cells have the capacity to generate their own rhythmicity that is governed by several levels of control including nervous, cytokine, and dietary influences as well as by immune cell products. This pumping rate appears to be increased during acute inflammation and may be depressed in its rate and forcefulness in disease. Interestingly our group reported that in intestinal

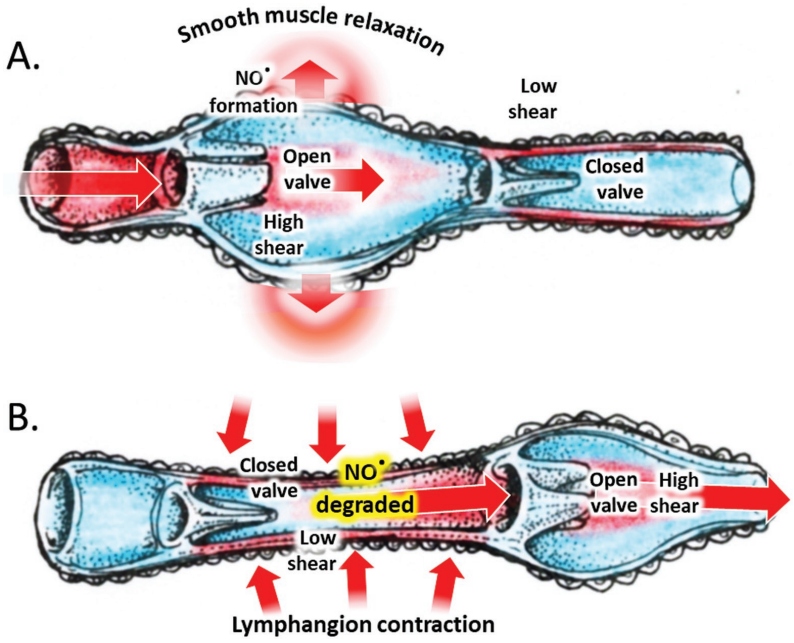


**FIGURE 3:** Regulation of lymphatic pumping. Events in A precede those in B, with lymphatic fluid being transported from the left to right. A) The lymphangion fills when the inlet valve is open, and the outflow valve is closed where tissue deformation may drive fluid into the lymphangion compartment. Fluid flow into the compartment increases flow and nitric oxide ($NO^{\bullet}$) formation, which helps to relax the lymphatic muscle cells in the lymphangion wall, further increasing the volume in the compartment and drawing in more interstitial fluid. B) In panel B, once the lymphangion has filled, back pressure closes the inlet valve, and fluid movement and shear stress drop such that $NO^{\bullet}$ is no longer formed. When $NO^{\bullet}$ concentrations drop, the lymphatic muscle cells can elicit contraction via spontaneous calcium influx triggered by mechanical, electrical, and ion flow mechanisms. This pumping rate can therefore be elevated by increased inflow of interstitial fluid and will reach maximum beyond which transport cannot be further increased by active pumping. This relative lymph flow rate can be increased during acute inflam-mation by fluid loading and can also be decreased during chronic inflammation by inflammatory lymphatic pump inhibition.

lymphatic muscle cells, interleukin-1b appears to lead to a paralysis of the tonic contraction of these cells by the induction of cyclooxygenase-2 (COX-2) with the evolution of smooth muscle relaxing prostaglandin $E_2$ [13]. This is an area under intense study by the Zawieja group at Texas A&M and by vonder Weid's group at University of Calgary, Canada.

Besides the effects of "extrinsic" factors acting on lymphangions to govern forward lymph propulsion, for instance the muscular contractions in the intestine which "massage" lymph forward, breathing movements that create gradients for lymphatic pumping through changes in

thoracic pressure, intrinsic factors—that is those that originate in the lymphangion at the molecular and biochemical levels also regulate the amount of force generated during tonic and how often lymphangions undergo phasic contractions in lymphangions.

Lymphangions are found in many tissues, with the mesenteric, intestinal and ovarian lymphangions as conventional examples of tissues displaying 'phasic' contractions [14]. Lymphangions support both tonic and phasic contractions and in many ways resemble spontaneously depolarizing cardiomyocytes that differ cardiac muscle tissues differing from skeletal and smooth muscle cells on several levels of signaling and regulation [12, 15, 16].

The rate at which lymphangions in the mesentery "beat" or pump spontaneously as a result of their inherent phasic contractions is enhanced by exposure to stimuli associated with food processing and is coordinated with food intake to allow transport of fats and fat-soluble materials as well as fluids. Hormones like cholecystokinin (released by enteroendocrine cells within the duodenum) and glucagon (produced by the pancreas) increase the rate of pumping of lymphangions. Lymphangions also release and react to mediators released by the endothelial lining of the lymphatics and products like Endothelin-1 (a potent vasoconstrictor). Endothelium-vasoconstrictor and endothelium-derived nitric oxide also influences phasic pumping [14]. Additionally, the outflow pressures from lymphatics provides an instantaneous stretch-mediated modulation of vessel wall tone that helps to further regulate intrinsic lymphangion pumping. This mechanism serves to adapt the flow in the entire network to accommodate increases in interstitial fluid load that arise due to feeding or physiological stimuli [17].

Several hormones and hormone-like substances (autocoids) like Bradykinin, the peptide substance-P, prostaglandin $E_1$, prostaglandin $F_{2\text{-}\alpha}$, 5-hydroxytryptamine, alpha-adrenergic agonists (norepinephrine), dopamine, and ATP which are released locally by tissues and by nerves terminating on or near lymphatics, have also been shown to alter the timing and forcefulness of lymphangion contractions. Lymphangion pumping can be negatively influenced by nitric oxide [18], as well as by anti-diuretic hormone (ADH), vasoactive intestinal peptide (VIP), prostacyclin (PGI2), acetylcholine, and by lipopolysaccharide [19].

The movement of fluid within the lymphatic lumen, the level of fluid shear experienced by the endothelial lining of the lymphangion, and the nitric oxide released by these cells in response to shear (and in response to mediators) appear to create a feedback system which tightly matches the intrinsic pumping characteristics of lymphangions [20] with the flow and biochemical environment experienced by the lymphatics. Interestingly, mast cell activation and the histamine released by these cells, an event which can be activated by beta-lactoglobulin, appears to diminish the pumping action of lymphangion [21].

Because the oxygen levels found in lymphatics is equivalent to or lower than that found in venous blood (10–35 mm Hg), oxygen levels may not be central to the control of pumping

of lymphangions. This low oxygen level found in the lymphatic environment may also help to maintain the unique lymphatic phenotype [22], but the low oxygen level does appear to provide sufficient energy to support lymphangion-pumping activity and raises the question as to whether lymphatics are vulnerable to ischemia-reperfusion injury. Although low oxygen levels may exist in and around lymphatics, oxidants such as hydrogen peroxide and $O_2^{\cdot}$—generated by inflammatory responses may be important signals to lymphatics, which in some cases inhibit lymphatic pumping [23]—an event that may help to limit the spread of pathogenic stimuli. Aging and disease also impair lymphatic pumping, and nitric oxide imbalances, elevation in nitrotyrosine, and age-related energy deficits may lead to weaker transport of lymph out of tissues, particularly when both age and disease are simultaneously present [24].

## 1.3 LYMPH FLOW AS DESCRIBED BY STARLING

Ernest Starling described the forces and relationships responsible for causing fluid movements between the vasculature and its surrounding interstitial spaces [25]. According to his now famous mathematical equation, microvascular ultrafiltration depends on the balance of two different pressures across the endothelial lining of the microvessel: hydrostatic pressure and osmotic pressure [26]. Classically, the vascular pressure gradient drives filtration of fluid from the vessels into the interstitium at the arterial side of capillary network and reabsorption in the venous end. However this concept has been recently reevaluated because the steady-state hydrostatic and osmotic pressures in most tissues results in a low level of fluid filtration from the capillary beds towards the interstitial fluid, regardless of the sides of the capillary network (arterial vs. venous). It is also important to keep in mind that this model requires that all forces and flows be in a steady-state and tend to be a simplified version of the real system [27], a set of conditions that is rarely observed.

According to Starling's model, under normal conditions, the amount of fluid leaving the capillary is identical to the lymph flow, and the tissue weight/volume remains constant. Lymph flow typically increases when capillary pressure increases, which in turn increases fluid filtration out of the vascular space. Initially, the magnitude of this increased lymph flow closely parallels increased capillary filtration. However at high filtration rates, the resulting change in lymph flow varies depending on the type of tissue (Figure 4). Lymph flow may plateau (meaning that it reaches a maximum possible flow rate) and maintain that flow rate despite any further increases in blood flow. This situation occurs in subcutaneous and muscle tissues [27]. However, in tissues like the intestines [28] and in the lungs [29], lymph flow actually diminishes at high capillary filtration rates. This reflects the opening of the overflow system, made up of mucosal membranes (in the intestines) and alveolar membranes (in the lungs), which acts to decrease

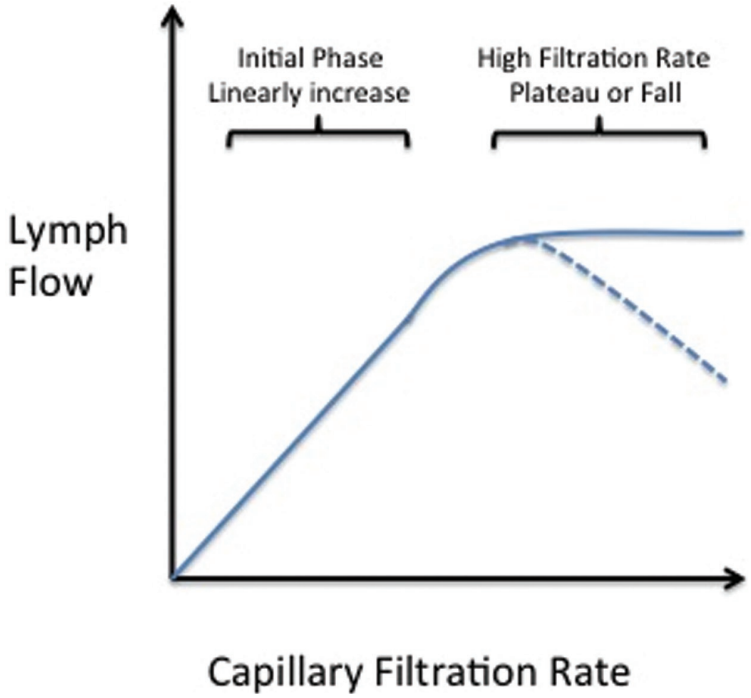


**FIGURE 4:** Lymph flow changes depend on the capillary filtration rate and vary anatomically. In some tissues such as subcutaneous lymphatic beds or muscle lymphatics flow plateaus at high filtration rates; in lymphatic networks in other organ systems like the intestines and lungs, overflow valves open, causing lymph flow to decrease at high filtration rates.

lymphatic filling pressure by alleviating the buildup of interstitial fluid pressure. The overflow system may cause the interstitium to behave like a balloon, and the resulting relationship between the radius and tension of the wall can be described using the Laplace equation. The Laplace equation describes the phenomenon in which an initial phase exists where a small change in volume results in a large change in pressure, followed by a second phase where the pressure decreases as the volume increases. The second phase continues until it reaches a critical volume, at which point the pressure increases again.

Under normal conditions, the interstitium is relatively dry and the compliance of this compartment is low. Consequently, small increases in interstitial fluid volume result in large increases in interstitial fluid pressure, with a correspondingly large increase in the formation of lymph—with the rise in interstitial pressure driving interstitial fluid into the initial lymphatics. As fluid accumulates in the interstitium, matrix components are expanded and disrupted, leading to a sudden increase in interstitial compliance. With the increased compliance, the capillary filtrate more readily accumulates in the interstitial spaces, the response of interstitial fluid pressure

to volume expansion is less robust, and lymph flow becomes less reactive to interstitial fluid accumulation, thus setting the stage for interstitial edema.

## 1.4 DEVELOPMENT OF LYMPHATICS

The formation and development of the lymphatic system is now better understood and truly fascinating. Once the primitive "cardinal veins" of the cardiovascular system form in the embryo, a subpopulation of venous progenitor cells is triggered to express the Prospero homeobox-1 (Prox-1) transcription factor, which acts as a key lymphatic fate-specifying signal (and also mediates development of several other tissue types as well such as the pancreas). Prox-1 can then trigger the upregulation of other secondary lymphatic-specifying and lineage-supporting genes in these progenitor cells to continue towards lymphatic "fate." It appears that the relative "dose" of Prox-1 is important for the full development of the appropriate number of lymphatic progenitors at this stage, without which too few lymphatics will form, resulting in a defective lymphatic, lymphedematous phenotype. Prox-1 expression is itself regulated by the transcription factor nuclear factor kappa-B (NF-κB), which is persistently expressed in lymphatic endothelial cells [30].

NF-κB is so intimately associated with lymphatic identity, that its continuous expression has even been used to mark and visualize lymphatic networks in several tissues such as the heart, bladder, lung and gastrointestinal tract [30]. In contrast, NF-κB activation is usually restricted to short periods following exposure to inflammatory stimuli in blood endothelial cells (BECs) (e.g., exposure to cytokines like TNF-a or IL-1b), and inflammatory activation of endothelial cells by NF-κB is especially prominent in venous endothelium [30]. However, despite constant expression of this inflammatory marker, lymphatic endothelial cells (LECs) do not have a chronically inflamed phenotype supporting greater rolling or adhesion of leukocytes.

NF-κB expression in lymphatic endothelial cells may be induced by VEGFR-3, and is responsible for the induction of the growth factor VEGF-C transcription [31]. It has even been suggested that defective lymphatics and development of the lymphedematous phenotype seen in subjects with VEGFR-3 missense mutations may, in large part, reflect deficiencies in this "normal" NF-κB signaling [32]. Therefore, it is not surprising that the persistent NF-κB expression in LECs has also been evaluated as a potential therapeutic target for controlling the growth of lymphatics in tumors, which is a major pathway for metastasis [33]. MTOR has also been proposed as a lymphatic target in some types of chemotherapy using rapamycin [34].

These Prox-1 expressing progenitors themselves appear to be under the control of COUP-TF II (which is expressed in most venous endothelium) and by the transcription factor SOX18 (SRY, sex determining region Y, box 18). SOX18 binds to and activates the Prox-1

**TABLE 1:** Lymphatic Endothelial Proteins Typically Used as "Markers" and their Chromosomal Locations

| LYMPHATIC MARKER/FACTOR | CHROMOSOMAL LOCATION |
|---|---|
| VEGFR-3 | 5 |
| LYVE-1 | 11 |
| FOXC2 | 16 |
| Prox-1 | 1 |
| Podoplanin | 1 |
| Reelin | 7 |
| VEGF-C | 4 |
| VEGF-D | X |

promoter [35]. Induction of SOX18 in BEC also drives LEC lineage development, whereas SOX18 suppression blocks lymphangiogenesis [35] and mutations in SOX18 produce the "ragged" mouse model; such mutations have also been linked with the phenotype seen in hypotrichosis-lymphedema-telangiectasia syndrome [36], a condition which also shows postnatal hair loss, vascular abnormalities, and lesions.

SOX18 appears to have enough functional overlap with SOX7 and SOX17 that it can rescue some mouse strains from the effects of *SOX18* gene deficiency (*SOX7* and *SOX17* are strain-specific modulators of the lymphangiogenic defects caused by *SOX18* dysfunction) [37].

Prox-1 expression is important in differentiating these progenitor cells into lymphatic endothelial cells (LECs), an observation which is supported by the induction of several lymphatic markers in cells forced to express Prox-1. These markers include vascular endothelial growth receptor-3 (VEGFR-3, also known as Flt-4), which is important in establishing a feedback loop for Prox-1 maintenance. This interaction appears to require the expression and appropriate processing of vascular endothelial growth factor-C, which binds to and activates VEGFR-3. In addition to these characteristic proteins, lymphatic endothelial cells also potently express the lymphatic vascular endothelial hyaluronan receptor (LYVE-)1, podoplanin, reelin [38], α1 and α9-integrins (Table 1).

## 1.5 LYMPHATIC ENDOTHELIAL CELLS

Lymphatic endothelial cells (LECs) are developmentally distinct but derived from venous endothelial cells. LECs share many structural and functional features with blood vascular endothelium, however to accomplish their unique functions, lymphatic networks are "deliberately" segregated and LECs are developmentally and programmatically distinct from blood endothelial cells (BECs) [39]. Lymphatic vessels differ from blood vessels in that they lack a true basement membrane and instead interact with fibrillin and Emilin-1 anchoring filaments which are important in both connecting lymphatics to tissue and transferring tension to these vessels to control lymphatic vessel distension, especially during edema development. Lymphatics also lack pericytes.

Unlike blood vessels, the cellular density of lymph is much lower than that of blood being composed of lymphocytes, antigen presenting cells, and monocytes. Moreover, erythrocytes and platelets are not normally present in lymphatics and the presence of either in the lymphatic lumen is considered pathological. Platelets actually participate in the separation of the blood and lymphatic vascular networks, so blood in the lymphatic system reflects a failure to segregate these networks [40]. Moreover, early investigations on junctional integrity suggested that LECs (especially in initial lymphatics), when compared to BECs, are less solute and cell restrictive, which allows the lymph microvessels to accommodate all materials to flow from the interstitial compartment into the lymphatic lumen [41].

Biochemical and molecular studies also reveal important distinctions between BEC and LEC. LECs abundantly express vascular endothelial growth factor (VEGFR)-3, podoplanin, Prox-1, LYVE-1, COUP-TF II, and reelin. Because these biomarkers are often not entirely specific for LEC (many markers are shared with other cell types such as venous endothelium and mesothelial cells), LEC fate and identity reflect the expression and cooperation between multiple LEC markers and determinants.

In addition to "resuming" proliferation to adaptively expand or reorganize lymphatics postnatally (often in response to prolonged inflammation), lymphatic networks can be expanded by recruiting cells originally derived from bone marrow, which become incorporated into the growing lymphatic vascular networks.

## 1.6 LYMPHATIC ENDOTHELIAL SPECIFIC MARKERS

Although LEC fate/identity programs are activated by Prox-1, Prox-1 also seems to depress blood endothelial-cell specifying transcripts to more fully discriminate these cell types pheno-

typically [39, 42, 43]. Such events are important in segregation of function and physical continuity. Interleukin-3 (IL-3) also appears to positively regulate Prox-1 activity and can induce the expression of LYVE-1 and podoplanin in BEC via mobilization of Prox-1 [44].

### 1.6.1 Prox-1

Prospero Homeobox-1 (Prox-1) is a homeobox transcription factor that is specifically expressed in only a subpopulation of endothelial cells in the embryonic vein. These cells give rise to the mammalian lymphatic system after budding from the cardinal vein. Prox-1 is required for both budding of these venous endothelial cells as well as their differentiation into lymphatic phenotype. Indeed, the embryos lacking Prox-1 gene expression display arrested budding at embryonic day 11.5 and fail to develop lymphatic vasculature. The endothelial cells that formed the initial bud also lack expression of any lymphatic proteins, including VEGFR-3 and LYVE-1, and express blood vascular genes instead, including laminin and CD34 [45]. Therefore, Prox-1 expression is indispensable for lymphatic fate determination of endothelial cells and is specifically expressed in lymphatic endothelial cells (LECs), but not in blood endothelial cells (BECs) [46].

Prox-1 is important in conferring lymphatic endothelial fate to venous progenitors by driving the expression of other downstream lymphatic-specific proteins such as vascular endothelial growth factor receptor-3 (VEGFR-3). Prox-1 and VEGFR-3 are both co-expressed in the LECs of lymphatic trunks and capillaries in 5-month-old human fetuses [47]. Prox-1 also induces the expression of integrin alpha-9, allowing integrin-dependent motility. On the contrary, Prox-1 expression is mutually exclusive with that of PAL-E, a blood vascular marker. Prox-1 is claimed to be the most reliable marker for LECs in both normal and pathologic tissues because of their constitutive expression in LECs of healthy adults, lymphedema patients, and lymphangioma patients, unlike some other markers (such as VEGFR-3), which tend to be less reliable due to the expression patterns in pathologic tissues [47]. Prox-1 is also closely linked with pancreatic beta cell development and there is a link between Prox-1 disturbances and diabetes. Interleukin-3 (IL-3) drives expression of Prox-1 and is an indirect lymphatic endothelial cell fate-determinant.

### 1.6.2 COUP-TFII

Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) is a transcription factor that acts as a co-regulator by interacting with various partners [48]. In the vasculature, it is only expressed in venous but not arterial endothelial cells, and the loss of COUP-TFII gene

results in the loss of venous identity and acquisition of arterial phenotype [49]. As mentioned previously, LECs sprout from venous ECs during embryonic development, and therefore venous identity is a prerequisite for LEC development. It is thus not surprising that COUP-TFII interacts with Prox1 to initiate and maintain LEC phenotypes [50]. COUP-TFII is required for Prox-1 induction, and after Prox-1 production, forms a heterodimer to induce a LEC fate [51]. Therefore, although COUP-TFII is not a lymphatic EC specific protein, it is crucial in LEC fate determination, mainly by inducing the expression of the ultimate LEC fate regulator, Prox-1.

### 1.6.3 VEGFR-3

Vascular endothelial growth factor receptor-3 (VEGFR-3) is a tyrosine kinase receptor that responds to vascular endothelial growth factors (VEGF) produced by a diverse group of cells, including endothelial, bone marrow and fibroblasts. VEGFs can be further divided into subtypes: -A, -C, -D. These different forms of VEGFs will be further explained in following sections. VEGFR-3 in particular binds to VEGF-A weakly and has much higher affinity for VEGF-C and VEGF-D, and plays an essential role in lymphangiogenesis. VEGFR-3 exists in the membrane as inactive monomers and are activated upon VEGF-C and VEGF-D binding, which promotes dimerization and autophosphorylation. Downstream signaling from VEGFR-3 activation leads to increased production of VEGF-C, creating a positive feedback loop that enhances and amplifies VEGFR-3 signaling. There is a secreted isoform of this receptor that functions as a decoy receptor for VEGF-C and VEGF-D to negatively regulate lymphangiogenesis to keep the process tightly modulated. Isoform 1 and isoform 2 are both transmembrane and bind VEGF-C and -D; however, isoform 2 is less efficient at activating the signaling cascade due to its lack of several phosphorylation sites resulting from its truncated C-terminus. VEGFR-3 can also regulate VEGFR-2 signaling by forming heterodimers.

As mentioned above, Prox-1 is responsible for the expression of VEGFR-3 in lymphatic endothelial cells. Downstream signaling of VEGFR-3 include MAPK1/ERK2, MAPK3/ERK1, MAPK8, JUN, and AKT1 pathways. Elevated VEGFR-3 expression is detected in LECs isolated from lymphangiomas [46], although these LECs display almost identical patterns of other lymphatic markers such as podoplanin, Prox-1, reelin and integrin alpha-9. Interestingly, these LECs from lymphangiomas also show decreased LYVE-1 expression. An A –> G mutation in VEGFR-3 is known to cause histidine-to-arginine substitution in the catalytic site of the proteins, which inhibits autophosphorylation of the receptor, leading to inactive VEGFR-3. This mutation is described as the cause of a congenital hereditary lymphedema, also known as Milroy disease, which is identified by a chronic swelling of limbs [36].

### 1.6.4 VEGF-C

Vascular endothelial growth factor-C (VEGF-C) is a glycoprotein growth factor that is secreted by several cell types and characterized by its ability to stimulate lymphangiogenesis by initiating LEC sprouting, proliferation, and survival [52]. Although structurally related to other VEGFs as well as to platelet derived growth factor (PDGF) family members, VEGF-C contains extra cysteine-rich motifs in its C-terminus [53]. Prior to binding to VEGFR-3, VEGF-C is proteolytically processed. Once bound to VEGFR-2 or -3, it causes tyrosine autophosphorylation and activation of its receptor. Mice overexpressing VEGF-C display hyperplasia specifically of the lymphatic network with apparently normal blood vessels [54]. Although VEGF-C predominantly binds to VEGFR-3, which is expressed mainly in the LECs, VEGF-C is also able bind and activate VEGFR-2, which is mainly expressed by blood vessels, suggesting a potential role for VEGF-C in the induction of angiogenesis as well [55]. VEGF-C–VEGFR-3 signaling also creates a chemoattractant gradient along which lymphocytes migrate from tissue to lymphatics [56].

Yonemura et al. [57] revealed that the number and diameters of VEGFR-3$^+$ lymphatic vessels was significantly higher in tumors that express high levels of VEGF-C (compared with those without exuberant VEGF-C expression), strongly supporting VEGF-C/VEGFR-3 signaling in the recruitment of tumor lymphatics. Because lymphatics represent an important conduit for metastatic spread, VEGF-C (and perhaps VEGF-D) mediated recruitment of lymphatics may be an important target in the suppression of metastasis.

Clinical studies of colorectal carcinomas show increased numbers of podoplanin-positive (D240$^+$) lymphatic vessels within these tumors, and this was shown to be a more accurate predictive and sensitive index of tumor invasion than the use of PECAM-1/CD31 (used as a marker or blood vessel density) or conventional histopathological analysis. Increased podoplanin$^+$/D240$^+$ lymphatic density in tumors has been significantly correlated with the extent of lymphatic invasion and metastases to lymph nodes, supporting the concept of lymphatic expansion as a channel leading to lymph nodes. Podoplanin/D240+ vessel density is also related to the extent of tumor invasiveness, and metastases of the vascular "pedicle" lymph node and liver [58]. Pancreatic cancer is also characterized by both VEGFR-3 and VEGF-C expression, which may indicate poorer outcomes reflecting metastasis [10].

### 1.6.5 VEGF-D

Vascular endothelial growth factor-D (VEGF-D) is a member of the VEGF/PDGF growth factor family and is structurally similar to VEGF-C [59]. Both growth factors are produced with long N- and C-terminal propeptides and display increased binding to VEGFR-2 and

VEGFR-3 following post-translational proteolytic cleavage and removal of the propeptides [60]. VEGF-C modified at its amino terminus can still bind VEGFR-2 but fails to bind VEGFR-3. Consequently, forms of VEGF-D modified in this fashion appear to be angiogenic but less specifically lymphangiogenic [61]. Unlike VEGF-A and -C, which are both necessary for normal embryonic vascular development, genetic deletion of VEGF-D does not produce an apparent phenotype [62]. Many tumors express abundant VEGF-C and -D that drive forms of tumor metastasis [63]. Conversely, blocking VEGF-C and -D signaling using soluble forms of VEGFR-3 traps can reduce tumor growth and the blockade of VEGF-C and -D have been intensively explored as cancer therapeutics [64].

### 1.6.6 LYVE-1

The lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 is homologous to the hyaluronan receptor CD44 and is frequently used as a lymphatic endothelial marker [65] based on its relatively greater expression in LEC compared to blood endothelium. LYVE-1 is an endocytotic receptor which interacts with hyaluronan, mediating its internalization in LEC. LECs process hyaluronan and move it between the luminal and abluminal cell faces, transporting hyaluronan from tissue compartments into lymph [66]. However, LYVE-1 knockout mice don't show an extreme phenotype [67] and are very similar to wild-type (normal) mice. Therefore, CD44 and LYVE-1 may be redundant and either alone might be dispensable for development, but knockouts for both exhibit less ability to withstand inflammation, which is consistent with LYVE-1 and CD44 fulfilling essential but overlapping roles during inflammation.

### 1.6.7 FOXC2

Forkhead box protein C2 (FOXC2), which is a member of the winged forkhead transcription factor family, is important specifically in the development of lymphatic system valves. Inborn errors involving FOXC2 cause lymph reflux and lymphedema. FOXC2-deficient mice fail to develop both lymphatic valves and also lack smooth muscle investment in their lymphatics and provide an important genetic model of the human condition "lymphedema distichiasis" (LDS) [68].

Such lymphatic valve forming endothelial cells are trigged to increase the expression of laminin alpha5, and to limit the expression of neuropilin-2 and LYVE-1 expression, which appear to be specific molecular signatures of cells within the lymphatic valve lineage [69]. These programming changes are thought to allow these cells to undergo specific structural changes that create valves.

FOXC2 is also known to regulate the Notch- and notch ligand pathways (specifically Delta-like 4 (Dll4), as well as the expressions of CXCR4, integrin β3, and Angiopoietin-2

(Ang-2), all molecules involved in angiogenesis [70]. Ang-2 is a major regulator of blood and lymphatic development and of inflammation [71], and is expressed in LECs [42, 72]. Ang-2-deficient mice exhibit gastrointestinal lymphodysplasia [73], and we have used this mouse model to investigate the role of defective lymphatics in chronic GI inflammation. In particular, we found that this strain is highly susceptible to developing more severe experimental colitis despite reduced leukocyte infiltration, lymphangiogenesis, and angiogenesis [74], supporting a protective role for lymphatic expansion during acute and chronic intestinal inflammation.

### 1.6.8 Podoplanin

Podoplanin is a 36- to 43-kDa mucin-type transmembrane O-glycoprotein that is specifically found in lymphatic endothelial cells, but also in pulmonary alveolar and podocytes, and participates in heart, lungs, and lymphatic system development. Podoplanin is not expressed by blood vascular endothelial cells. Consequently, podoplanin has been widely used as a "selective" marker for LECs [75]. Sialylated O-glycans on the extracellular domain of podoplanin appear to protect the podoplanin against proteolysis by enzymes present in lymph [76].

### 1.6.9 Reelin

In addition to the passive tissue contractions responsible for driving the propulsion of lymph by lymphangions, contractions of lymphatic smooth muscle cells (LSMC) that surround the collecting lymphatic vessels are responsible for a large proportion of the motive force underlying efficient lymphatic transport. One of the proteins now thought to be mainly responsible for the correct functioning of LSMC in lymphatic function and morphogenesis is Reelin, which was discovered in 2012.

Reelin is an extracellular matrix glycoprotein that was previously thought to be solely involved in the development of the central nervous system (CNS). In the CNS, mutation in the reelin gene prevents normal development of the cerebral cortex and disrupts proper cognitive development. This results in a condition known as Norman-Roberts syndrome (NRS), which is a rare type of lissencephaly, a set of brain disorders characterized by smooth-appearing brains [77]. NRS patients display congenital lymphedema and cerebellar hypoplasia as well as reduced cerebellar volume. Another CNS disorder that results from a disruption of the reelin signaling pathway is VLDLR (Very Low Density Lipoprotein Receptor)-associated cerebellar hypoplasia, which is caused by a mutation in one of the reelin receptors, VLDLR.

Reelin has also been implicated in regulating the development of the lymphatic vasculature [78]. It was found that mice lacking reelin not only displayed impaired lymphatic function,

but also showed abnormal collecting lymphatic morphology. Specifically, these mice displayed reduced numbers of LSMCs in collecting lymphatic vessels, demonstrating the importance of reelin in mediating smooth muscle cell (SMC) recruitment to lymphatic vessels. This reelin-mediated SMC recruitment involves an autocrine "feed-forward" mechanism that works in the following fashion: LSMC recruitment induces both the release and proteolytic activation of Reelin from endothelial cells. This activated reelin, in turn, stimulates lymphatic endothelial cells to increase their expression of the SMC recruitment factor, monocyte chemotactic protein-1 (MCP-1). Reelin receptors include VLDLR and ApoER2 (apolipoprotein E receptor 2) as well as integrin alpha-3/beta-1, all of which are expressed by endothelial cells. Because loss of reelin reduces leukocyte binding [79], reelin may also play a role in leukocyte adhesion and trafficking in lymphatics. Interestingly, NRS is also characterized by congenital lymphedema and may be considered within the spectrum of lymphedema. Although involved in both lymphatics and the CNS, it is also currently unclear whether reelin is related to CNS lymphatics or to glymphatic function.

## 1.7 BLOOD VS. LYMPHATIC ENDOTHELIAL SPECIFIC MARKERS

There are also markers that are "specific" for blood endothelial cells. These include pathologische anatomie Leiden-endothelium(PAL-E), plasmalemmal vesicle-1 (PLVAP) [30], α5- and β5-integrin, ICAM-1, N-cadherin, claudin-7, vinculin, CD34, the hyaluorinic acid receptor CD44 (a LYVE homolog), endoglin, interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), collagens 8A1, 6A1, and 1A2, laminin-γ2, uPA (urokinase plasminogen activator), matrix metalloproteinase-1 (MMP-1), signal transducer and activator-6 (STAT6), UFO/axl, and CXCR4 [42]. Selectins, ICAM-1, VCAM-1, and MAdCAM-1 represent common biochemical markers which are shared between blood vascular and lymphatic endothelial cells, and thus are not considered to be "discriminating" markers.

Lymphatic endothelial cells have also been shown to express multiple integrins including α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, αvβ3, and α9β1 [80] (not all of these however are specific for lymphatic endothelium). Lymphatic endothelial cells also bind to fibrillin and Emilin-1-containing anchoring filaments through several of the integrins containing adhesive mechanisms. Interestingly, α4β1 expressed by lymphatics also seems to be an important mechanism underlying the metastasis of tumor cells through lymphatics, an event which involves the activity of phosphatidylinositol-3 kinase [81], which may indicate that PI3 kinase inhibitors could reveal an additional lymphatogenous metastatic target for cancer therapy.

## 1.8 BLOOD AND LYMPHATIC VESSEL SEPARATION

Blood and lymphatic vascular networks are separate and must remain so to accomplish their different functions or they will fuse, leading to lymphatic dysfunction. This separation is mediated by signaling modules on platelets and lymphatic endothelial cells.

Podoplanin-deficient mice that have fused blood and lymphatic vessels die as embryos, and exhibit blood-filled lymphatic vessels and severe edema [82]. Blocking podoplanin with anti-podoplanin antibody also blocks lymphangiogenesis under inflammatory conditions [83], showing podoplanin's importance developmentally and postnatally.

It has been shown that endothelial expressed mucin-type O-glycans maintain essential separation of blood/lymphatic circuits and prevent development of fatty liver disease. This O-glycan modification of podoplanin depends on the activity of the glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (C1galt1) gene, where C1galt1 O-glycan deficiency leads to blood/lymphatic misconnections [84] and also provokes fatty liver disease. This podoplanin modification is also essential for allowing podoplanin to interact with platelets, which is an important signal in blood/lymph separation [85].

The molecular signals "Syk" (spleen tyrosine kinase) and its target, SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), are expressed by platelets (and also by bone marrow derived endothelial precursors) and provide important signals following contact with lymphatic endothelial cell expressed podoplanin, which act to maintain the separation of the blood and lymphatic vascular compartments [86]. Expression of podoplanin within lymphatics triggers platelet activation by binding to platelet C-type lectinlike receptor 2 (CLEC-2), which is clustered, leading to the activation of SYK through phosphorylation of hemITAM. This in turn activates complexes including SLP-76 and PLCγ2 which stimulate platelet aggregation and prevent blood to lymphatic vessel fusion [87]. Similarly, Rac1 expression is also involved with blood and lymphatic vessel separation, and endothelial loss of this GTPase causes a failure of lymphatics to maintain separation from blood vessels leading to fusion [40].

Spreds-1 and -2 are negative regulators of VEGF-C/VEGFR-3 receptor signaling that may play a role in LEC specification, development, and blood-lymph separation [88]. When deleted, Spreds knockout cells showed fusion of blood and lymphatic networks. Without Spreds, lymphatic endothelium were highly responsive to VEGFR-3 signialing; Spreds therefore negatively regulate lymphatic (but not blood vascular) activation. Consequently, appropriate lymphatic growth restriction may also be involved in maintaining separation of these networks.

One more soluble factor that helps to control the separation of blood and lymphatic networks in the small intestine is angiopoietin-like factor-4 (ALF-4), also known as fasting-induced

adipose factor (FIAF). FIAF is increased in adipose tissue during fasting, ischemia, and in response to anti-diabetic thiazolidinediones [89]. Conversely, FIAF is decreased by fat feeding and by a decrease of *Bifidobacteria* [90] in the gut microbiome, a condition found in post-antibiotic induced diarrhea, inflammatory bowel disease, irritable bowel syndrome, and obesity.

Mice with FIAF knocked out can exhibit fused blood and lymphatic vessels, particularly in the small intestine. FIAF controls the expression of Prox-1 [91] and also controls Syk (see above) [91]. The mechanism of ALF-4 in Prox-1 regulated intestinal lymphatic homeostasis provides links of Prox-1 with the normal control of insulin release. ALF-4 derived from the hypothalamus also regulates energy balance (see below) [92].

Integrins, such as a5b1 for example, also appear to be involved in maintaining the separation between blood and lymphatic networks, as they are also important regulators of lymphovenous valve integrity [93]. The mechanism for a5b1 involves its participation in maintaining appropriate responses to Angiopoietin-1, which controls vessel growth control, stabilization, and functional "maturity."

### 1.8.1 Other Regulators of Lymphatic Patterning

Many signaling and structural proteins, although not specifically expressed by lymphatic endothelial cells, modulate lymphatic formation and patterning. Lymphatic networks express "Ephrins," a family of receptor-protein tyrosine kinases that control proliferation and differentiation. Expression of Ephrin b2 and b4 control the recruitment of smooth muscle and in lymphatics, and Ephrin b2 appears to participate in the creation and differentiation of lymphatic capillaries and collectors from existing lymphatic networks. Interestingly, the specific deletion of Ephrin b2 in lymphatics produced a phenotype that has defects similar to that seen in some forms of hereditary lymphedema. However genetic elimination of Ephrin b2 in both blood and lymphatic endothelial cells results in malformed vessels and hemorrhage [89], which could reflect the restriction to lymphatics, and there is not compelling evidence for Ephrin b2 in congenital lymphedema.

The Notch signaling pathway, a type of lock and key signal requiring two-cell contact, is also expressed in lymphatic endothelial cells, where the Notch 1 and 4 forms are expressed; Notch may play a role in lymphatic patterning and maintaining lymphatic structure. This type of regulation may be important in lymphatics because of the effect Notch signaling has on the expression of both Ephrin b2 and VEGFR-3 [94].

Some other proteins that appear to be important in establishing the unique lymphatic phenotype include "Elk" or Ets-like gene 1. When Elk is suppressed, the appearance of lymph

in the thoracic cavity is seen (chylothorax), and thoracic lymphatic vessels are congested but peripheral edema (lymphedema) isn't apparent, thus its effects may be restricted to abdominal/thoracic compartments. When the lymphatic endothelial-specific protein Podoplanin is suppressed, the resulting abnormal lymphatic networks produce a type of hereditary lymphedema which can be seen in the skin, legs, and neck, and also affects the intestine where lymphangiectasia is apparent, and thus its influence is more extensive.

In the initial lymphatics, where interstitial fluid is collected, the lymphatic endothelial cells are much thinner compared to blood vascular endothelial cells; lymphatics also have almost no basal lamina and have differently assembled junctions and lack fenestrations. Because of this loose arrangement, fat droplets (chylomicrons) as large as 620 nm can passively penetrate into the lymphatic lumen with little difficulty. This is because the initial lymphatic endothelial cells have "flaps," whose overlapping creates a kind of one-way valve resembling oak leaves and permits these large complexes to enter lymphatics, going around the cells instead of through them as is seen with "fenestrations" in endocrine glands and in the kidney. When interstitial fluid pressure increases, such as during acute inflammation, the expansion of the tissue creates tension on oxytalan, elastin, and elaunin fibers which tug on the flaps, opening these lymphatic valves to increase interstitial fluid collection [41, 72].

These flap-like cells function as microscopic valves that give way to periodically spaced junctions which can be seen in the next region, the so-called "collecting" lymphatics [95]. These intermittently fastened, button-like junctions contain vascular endothelial (VE)-cadherin, occludin, claudin-5, zonula occludens (ZO)-1, junctional adhesion molecule-A (JAM-A) and endothelial specific molecule-1 (ESM-1). Normally these molecules are also found in blood vessel endothelium, but these are usually continuous around the perimeter of the cells in blood endothelium where they may create a more restrictive barrier rather than the periodic pattern in lymphatics.

Smooth muscle investment in collecting lymphatics is triggered by PDGF-b [96] released by lymphatic collecting endothelial cells, which causes recruitment of muscle cells, a process that begins during embryogenesis and continues throughout life. One more condition that may modulate lymphatic identity is hypoxia, when lymphatics experience an oxygen concentration at or below that found in the venous circulation. Such hypoxia may induce Notch 4 which suppresses VEGFR-2 but increases VEGFR-3 signaling, which in turn powerfully drives endothelial identity towards lymphatic specification.

Another factor which may contributes to lymphatic response to growth factors driving specification is Neuropilin-2 (NRP-2). In lymphatic endothelium NRP-2 is a "co-receptor" that captures VEGFs to bring them into close approach with VEGFR-3 to induce lymphatic activation [33].

Interestingly, NRP-2 is downregulated during the formation of lymphatic endothelial valves. In these NRP-2 down-regulated lymphatic-valve forming endothelium, LYVE-1 is also suppressed, whereas laminin a5 is increased. These molecules therefore represent presumptive molecular markers of lymphatic valves [69] that also help drive cell-specific patterning to form these structures.

Although these events all take place during development when lymphatics arise from venous primordia, lymphangiogenesis can of course also occur postnatally, and lymphatic networks can expand from existing networks and from macrophage-like lymphatic progenitors [97]. Both forms of lymphatic development can be potently controlled by members of the VEGF family (-C and -D), local factors (oxygen tension), as well as biomechanical forces (e.g., flow and matrix metalloproteinases). For example, lymphatic formation during wound healing seems to be controlled by factors that control the reestablishment of interstitial flow [98], where MMP-2 and -3 flow through tissues to create fluid channels which become populated by lymphatic endothelium that form lymphatic conduits.

Interestingly, another matrix metalloproteinase (MMP), MMP-9, appears to also be involved in lymphatic recovery, as MMP-9 knockout mice have persistent lymphedema following surgeries, which strongly suggests that MMP-9 is needed for lymphatic regeneration after wound healing [99].

Lymphatic remodeling after injury may also be indirectly controlled by nitric oxide (NO), which may govern the amount and types of growth factors as well as the blood vascular angiogenesis that proceeds in parallel with lymphangiogenesis [98]. Although these factors may drive the development of functional lymphatics, several other molecular classes, particularly inflammatory cytokines and lipopolysaccharide, can activate an inflammatory lymphatic phenotype or impair lymphatic function [100]. Transforming growth factor-β is involved with lymphatic remodeling [101] and may represent a target for lymphatic regeneration after surgical injury [102].

### 1.8.2 Lymphatic Barrier Function

Barrier function in lymphatic endothelium may share several structural and functional mechanisms with blood endothelium. The lymphatic endothelial barrier function is known to be regulated by several mediators including adrenomedullin (AM), which organizes ZO-1 and VE-cadherin at cell junction membrane to limit trans-lymphatic exchange [103]. The role played by AM in the regulation of lymphatic exchange is however debatable. AM may be involved in the development of vascular networks [104], including driving lymphangiogenesis; AM may also represent a tool to suppress lymphedema [105].

We previously reported that proinflammatory cytokines disturbed several lymphatic endothelial functions, which may underlie inflammatory responses *in vivo* [106]. For example, we observed that tumor necrosis factor-a, interleukin-1b, and IFN-g all induced mouse LEC surface expression of ICAM-1, VCAM-1, MAdCAM-1, and E-selectin; tumor necrosis factor-α, interleukin-1 beta, and IFN-gamma induced ECAMs (but not MAdCAM-1) in human LECs. Intriguingly, LEC proliferation was increased by interleukin-1 beta, but decreased by IFN-gamma and tumor necrosis factor-α. In human LECs, tumor necrosis factor-α induced proliferation, whereas IFN-gamma decreased proliferation. All cytokines tested suppressed capillary formation in Matrigel in mouse and human LEC. Tumor necrosis factor-α and interleukin-1 beta decreased barrier function in both mouse and human LEC. IFN-gamma did not affect mouse barrier function, but enhanced human barrier.

Cromer et al. 2014 showed that inflammatory cytokines and lipopolysaccharides (LPS) can increase transmonolayer exchange across cultured lymphatic endothelium through several mechanisms including nitric oxide and overall expression of junctional components [107]. It was noted that interferon-gamma produced the greatest increase in permeability (2.5-fold normal), with more modest increases induced by TNF-α (tumor necrosis factor-alpha), interleukin-6, lipopolysaccharide, and interleukin-1b. Interestingly, barrier disturbances produced by tumor necrosis factor-alpha, interleukin-6, and interferon-gamma appear to be mediated at least in part via formation of nitric oxide. Specifically, the serine19 phosphorylation of myosin light chain 20 appears to be a target of cytokine (tumor necrosis factor-a, interleukin-6 and IFN-g) induced nitric oxide signaling, which also influenced relative abundance of VE-cadherin and beta-catenin. Signaling in response to mediators may also be powerfully affected by the pathophysiological state. For example, nitric oxide appears to be an important regulator of lymphatic barrier, which is also influenced by diabetes. Scallan et al. 2015 demonstrated a lymphovascular defect in type 2 diabetes, where lymphatic solute permeability is increased by low nitric oxide levels [108]. Normally, permeability mediators like platelet-activating factor and VEGF-A stimulate endothelial nitric oxide synthase (eNOS) to reorganize cytoskeletal and junctional elements, which is seen under non-diabetic conditions.

Lymphatic permeability was found to be >100-fold greater in diabetic ("db/db") leptin receptor-deficient mice than wild-type mice. In these mice, NO did not increase permeability, but paradoxically, protected the barrier. This elevated permeability was attenuated by arginine (an NO-precursor) and was reversed by the arginine/NO antagonist L-NAME. Therefore, when vascular permeability is increased by diabetes, NO/cGMP may maintain the lymphatic barrier via phosphodiesterase 3 (PDE3). PDE3 degrades cAMP more efficiently than cGMP and is also suppressed by cGMP. Under these conditions, NO increases cGMP and inhibits

PDE3 and blocks cAMP degradation. Diabetes produces a strong cAMP suppression, which increases permeability in this condition. Therefore, PDE3 inhibitors such as cilostamide or arginine supplements may have clinical applications in relieving diabetes-associated lymphatic barrier disturbances [109].

## 1.9 LYMPHATICS AND LEUKOCYTE TRAFFICKING

The lymphatic system serves as an essential conduit for the recirculation of lymphocytes, which travel from blood into tissues then to lymph nodes, or accessory lymphoid tissues, then return back to the circulation. LECs, just like BECs, express endothelial adhesion molecules and chemokines on the surfaces that support their interactions with activated immune cells. For example, SLC/CCL21 (a ligand for the receptor CCR7) expression is induced on LECs in response to several inflammatory stimuli such as LPS or VEGF-C [110]. The increased SLC/CCL21 expression on the LEC apical surface leads to the retention of CCR7-positive naïve Th1 T-cells and dendritic cells and is commonly found in Crohn's disease, specifically within mesenteric lymph nodes [111].

Increased SLC/CCL21 expression is also found in LECs of the non-obese diabetic pancreas [89], suggesting that lymphatics may be involved in inflammatory pancreatic injury. CCL20 and CCL21 expressed by LECs are also important for lymphatic immune surveillance, particularly by facilitating CCR6+/7+ leukocyte trafficking [112]. Hyaluronic acid expressed by lymphatics may also be important for directing leukocyte trafficking into the lymphatics, possibly influenced by LYVE-1 interactions with hyaluronan.

CHAPTER 2

# Lymphatics in Different Organ Systems

## 2.1 THE BRAIN

For decades the brain has been believed to function normally without conventional lymphatic drainage. However, in recent years, new findings have suggested that lymphatic drainages do occur in the central nervous system (CNS). Three major routes of lymphatic clearance in the brain have been proposed: 1) basement membrane (BM)-based perivascular pathway, 2) brain-wide glymphatic pathway, and 3) the meningeal pathway.

Perivascular CNS lymphatic drainage follows a unique route, unlike classical lymphatic vessel structures found elsewhere in the body. Its drainage pathways have been studied using different tracer dyes such as Indian ink [113] as well as radioactive tracers [114] and fluorescent tracers [115]. The Weller-Carare group has proposed that CNS lymphatic drainage occurs via perivascular pathways along the BM of cerebral arteries [115]. Interestingly, lymph flows in the opposite direction from blood flow, possibly resulting from pulsatile flow in arteries; therefore it is suggested that conditions that reduce pulsatility, such as aging, causes impaired elimination of solutes and waste in CNS lymph [116].

Another set of lymphatic drainage routes in the brain has been suggested by Nedergaard's group [117]. Referred to as the "glymphatic" system, this brain-wide paravascular pathway allows the exchange of cerebrospinal fluid (CSF) and interstitial fluid (ISF) via aquaporin-4 (AQP-4) water channels expressed on glial cells (hence the name "glymphatic") and aids in efficient clearance of waste and toxic solutes, such as amyloid-β (Aβ), from the brain. The convective exchange involves the influx of CSF in the arteries and efflux of ISF along the veins [118]. CSF influx is driven in part by arterial pulse, and surprisingly, is significantly reduced in the conscious state compared to sleeping or anesthetized state, resulting in impaired clearance of neural metabolic waste during wakefulness [119].

In 2015, Aspelund et al. [120] and Louveau et al. [121] independently characterized brain lymphatic vessels in the meninges with lymphatic valves and lined with lymphatic endothelial

cells positive for LYVE-1, podoplanin and prox-1. Therefore, these are more like conventional lymphatic vessels and function as a common path for perivascular ISF (Weller's model) as well as CSF/ISF (glymphatic model). Malfunction of the CNS lymphatic system correlates with neurological diseases including multiple sclerosis, Alzheimer's disease, and exacerbated stroke outcomes.

## 2.2 THE EYE

Transport of lymph formed in the eye remains a complex topic and is still not completely settled. To maintain optical clarity, the eye limits the expression of both blood and lymph vessels in the cornea, lens, and vitreous humor. These tissues are normally avascular and the induction of vascular networks almost always indicates a pathological process. Following exposure to injury, infection, or inflammation, the cornea will undergo lymphangiogenesis (and this can also occur in the iris, ciliary body, and conjunctiva). The cornea has therefore been used as an ideal model in which to study induced development of such networks, such as in tumor vessel recruitment. Conversely, the tear glands, the conjunctiva (white), limbus (cornea-scleral border), and the optic nerve are all densely supplied with lymphatics. The corneal limbal lymphatics also appear to have some unusual properties in that they express LYVE-1 discontinuously, which may confer valve-like properties in these structures [122]. Similarly, the iris and retina have no lymphatics but lymphatics are present in the ciliary body and the white of the eye (sclera). This lymphangiogenesis appears to be independent of blood vessel angiogenesis. The lack of blood and lymphatic vessels in the cornea appears to reflect the production of soluble VEGFR-2 and VEGFR-3, which act as VEGFs-A, C and D "sinks" that isolate these growth factors in tissues to prevent growth of these vessels, both blood and lymphatic [123, 124].

Lymphangiogenesis in the cornea has been linked to the failure of corneal transplants, with increased immune cell trafficking through these networks predicting poorer outcomes [122]. This connection appears to indicate that intra-lymphatic transport of antigens and antigen presenting cells (APC) from these areas drives activation of the immune system, which provokes tissue rejection. It has also been shown that corneal transplants do better when lymphatics are suppressed, which prolongs and stabilizes these grafts. Preventing the development of lymphatic vessels using lymphatic suppressing agents like the VEGFR-3 kinase blocker "MAZ-51" might thus improve survival of corneal [125] and perhaps other grafts.

The conjunctiva has both clearly defined blood and lymphatic vessels, and lymphatics dilate and remodel during conjunctivitis [126]. Using Trypan blue tracer injection studies, Singh et al. have described an important lymphatic network under the conjunctiva linked to scleral

lymphatics that communicates fluid formed in the aqueous humor. Nakao et al., 2012 suggest that tissues supporting outflow from the aqueous humor may at least have properties that are similar to those of true lymphatic vessels, in that they conduct immune cells and antigens to lymph nodes. The Schlemm's canal (SC) clears fluid from the aqueous humor [127] and has many lymphatic features. Podoplanin has been found in lymphatic vessels in the conjunctiva, and apparently in association with the conjunctival and corneal epithelia, corneal endothelium, and the sheath of the optic nerve [128] as well as near the ciliary muscle. It is unclear if Schlemm's canal "endothelial cells" express podoplanin. These intermediate phenotype endothelial cells do express the lymphatic transcription factor FOXC2, vascular endothelial (VE)-cadherin, alpha-9 integrin, CCL21, and also express VEGFR-3, which allows them to respond to VEGF-C and -D (which has been considered for glaucoma treatment) [129]. Like true lymphatics, SC cells express the transcription factor Prox1 and SC is formed by in-growth of vein cells, developmentally similar to lymphatics [130], however, conventional lymphatics are not described. Angiopoietin-1 and -2 also appear to participate in development of lymphatic vessels in the eye [131].

### 2.2.1 The Lungs

During normal breathing, the lungs are continually subjected to microparticulates and noxious materials such as microbes and viruses in the 350 cubic feet of air that are inhaled daily. The interstitial spaces between the alveoli is constantly experiencing shifts in osmotic, pneumatic, and hydrostatic forces and needs to tightly control transcapillary fluid and protein balances during lung inflation to maintain normal oxygen and carbon dioxide exchange [132]. This is accomplished by the pulmonary lymphatics, which act to keep the alveoli and the gas exchange areas free of excess fluid to prevent interstitial expansion or alveolar filling with fluid. These lymphatics also clear toxic materials. Lungs of premature infants may lack VEGF-C which would normally maintain or enhance lung fluid drainage, potentially intensifying edema and limiting lung inflation [133]. Sarcoidosis, another condition affecting the lung, is also characterized by granulomatous lymphatics which may also impair lung fluid balance [133]. Normally, lung lymphatics are normally partially collapsed, but can swell open when additional fluid clearance is needed [133]. Pulmonary lymphatic filling can vary depending on the state of edema, with the largest increases noted around pulmonary venous sphincters [134].

Pulmonary pre-lymphatics are found in the visceral pleura and expand to clear lymphatic fluid during edema. These pre-lymphatics merge into reservoir lymphatic lacunae, which have a more network-like appearance and connect to tubular conduit lymphatics. Both are considered "initial" lymphatics [135]. Saccular lymphatics are another type of lymphatic vessel that

surrounds the airways of the lungs. Pulmonary lymphatics are sensitive to high oxygen concentrations and can change diameter accordingly. For example, after being exposed to high (85%) oxygen for 7 days, rats show extensive expansion of saccular lymphatic networks which is resolved after restoration to normal air for two weeks [135].

Pulmonary lymphatics also expand in response to chronic airway inflammation (e.g., with infection) which leads to more numerous and persistent lung lymphatics. This lymphangiogenesis is largely dependent on VEGF-C, VEGF-D, and VEGFR-3 signaling, as VEGFR-3 inhibition leads to loss of lymphatic vessels without changes in blood vessels. The roles of lymphatics in asthma suggest that airway edema may be mediated by poorly functioning lymphatics.

### 2.2.2 Tonsils

Located at the base of the tongue and throat, the tonsils are important secondary lymphoid organs and are part of the gut-associated lymphoid tissue (GALT) tissue. The tonsils are arranged in a tissue "ring," known as Waldeyer's ring, and has four component divisions: the pharyngeal or adenoidal tonsil, two tubal and two palatine tonsils, and the lingual tonsils. Tonsils are the initial line of defense against ingested or inhaled pathogens, and tonsils have an abundant supply of B- and T-lymphocytes, and the tonsils may represent a site where T cells are generated and mature [136]. Although the tonsils have a dense supply of lymphatics, they appear to lack afferent lymphatics. Tonsillar lymphatics drain from lymph nodes in the back of the throat (medial retropharyngeal) to the jugular lymphatic duct, merging with the blood at the junction with the brachiocephalic veins. Tonsillar lymphatic obstruction produces tonsillitis (tonsillar lymphangitis), which may require surgical removal of the tonsils.

### 2.2.3 Salivary Glands

Salivary glands have an extremely high lymph flow when compared to other regions of the intestinal tract which is related to the requirement of saliva from interstitial fluid. Two main types of lymphatics are found in the salivary capsule and salivary duct blood vessel-associated lymphatics. Interestingly, the expression of the lymphatic selective marker podoplanin is low in the acini of the parotid salivary gland, but is present in submandibular and sublingual glands [137]. Podoplanin-positive lymphatics are more abundant in sublingual glands compared to submandibular salivary glands. Salivary glands also actively secrete the growth factor and cytokine VEGF-A [138], which reaches the saliva where it may increase blood flow and solute permeability of the blood capillaries of the salivary glands. Because VEGF-A appears to enhance interstitial fluid delivery

in this way, it may be intimately involved in saliva formation. Salivary VEGF-A may also help to induce and maintain blood and lymphatic vessels after surface injury in the intestine, exerting trophic/restorative effects on the GI lining and blood vessels to preserve epithelial integrity [138]. The relationship between apthous (mouth) ulcers and low levels of salivary VEGF-A are consistent with this idea [139]. We have detected lymphangiogenic VEGF-C and -D in saliva (unpublished observations) which may also contribute to lymphatic maintenance following breach of the epithelial surface and communication of these growth factors to the underlying interstitium.

### 2.2.4 Stomach

Gastric (stomach) lymphatic flow begins within sinuses next to the mucus forming pyloric gland in the terminal region of the stomach. Gastric lymph flow is approximately 0.06 ml/min per 100 g under normal conditions. These sinuses join to merge the lymphatic networks of the gastric muscularis mucosa and the submucosal layers. The lymphatics of the gastric mucosa are far from the lining, arising near the muscularis mucosa, and the upper regions of the mucosa lack lymphatics [140]. The mucosal thinning that occurs in atrophic gastritis makes these lymphatics appear closer to the surface lining [141]. Gastric lymphatics usually follow arteries anatomically and frequently drain into lymph nodes along the arteries they follow. Gastric lymph ultimately drain into pre-aortic/"celiac" lymph nodes in front of the aorta. At birth, submucosal and subserosal gastric lymphatics in the stomach rapidly expand in response to food ingestion and is most apparent in the stomach's lesser curvature. The presence of lymphatic valves in the walls of the stomach have also been revealed using staining for 5'-nucleotidase, an early enzymatic method for marking lymphatics.

### 2.2.5 Intestinal Lymphatics

Because the gastrointestinal tract has an immense surface (>200 m) to facilitate the absorption of nutrients and fluid, lymphatic networks in the intestines play critical roles in both interstitial fluid and fat transport. The gastrointestinal lumen (especially that of the large intestine) contains, compartmentalizes, and is densely colonized by the ~$10^{14}$ bacterial commensals which make up the intestinal microbiome (for both good and bad), and therefore gastrointestinal lymphatics play critical roles in helping to respond appropriately to these diverse constituents in support of normal mucosal immunity. Lymph flow in the small bowel is reported as 0.045 ml/min per 100 g which is 3 times greater than the large bowel (0.015 mL/min/100 g) and reflects the nutrient absorption carried out here.

More than half of all lymph formed daily is created in the GI tract. Lymph flow in the gastrointestinal tract varies greatly. Lymph flow in the liver is 0.05 mL/min/100 g, whereas that of the salivary gland is much higher at approximately 0.14 mL/min/100 g (see section on salivary glands). In addition to normal increases in lymphatic flow that can be seen following feeding (originally recognized by Aselli), several forms of intestinal pathology can increase lymph flow especially acute inflammation. Conversely, chronic intestinal inflammation such as Crohn's disease may result in a lymphangitis and a mismatch of lymph formation and clearance with blood flow. Intestinal obstruction can also trigger inflammation which increases lymphatic flow [8].

### 2.2.6 Lymphatics within the Small Intestine

Lipid absorption in the small intestine is accomplished at the central lacteals in the villi, which are tube-like projections found on the surface of the intestine which dramatically increase surface area for absorption. Lacteals in the small intestinal villi are found in the center of every villus. The lacteals are usually 50 microns away from the basement membrane [142]. Lacteals are important for carrying fat-laden filtrates into the lymphatic networks, into which these lacteals merge. These jejunal and ileal lymphatic plexuses form webs known as the peri-glandular and sub-mucosal plexuses. Lymph from the jejunum and ileum drains from mesenteric nodes into the celiac lymph nodes, from there to intestinal lymphatic trunks, then to the confluence of the abdominal lymph trunks, and ultimately the thoracic duct.

Intestinal lymphatics are critical for maintaining gut homeostasis by serving as the central conduit for the clearance of fluid, plasma proteins, and blood cells entering the intestinal interstitium. Lymphatics of the small intestine transport lipids that are absorbed, packaged, and released by enterocytes in the small bowel [143]. During embryogenesis, small intestine lymphatics form from mesenteric lymphatics by branching and extending, but not from intestinal mesoderm [144]. Despite the involvement of LYVE-1+ macrophages that activate VEGFR-3, the important observation that mice lacking LYVE-1 expression do not differ phenotypically [145] suggests that redundant pathways may exist, substituting for LYVE-1+ macrophages. Lymphatics in the small intestine collect fluid that penetrates into the mucosal interstitium [146]. The proportion of lymphatic fluid clearance varies over the intestine, possibly reflecting different requirements for transport functions that regulate lymphatic density (and transport capacity). This depends on both anatomic and species differences. The amount of interstitial fluid which can be handled by the lymphatic network of the small intestine greatly exceeds that of the large intestine.

There are several levels of lymphatic networks within the mucosa, submucosa, and muscle layers of the small intestine. Perhaps the best known component of the small intestinal lymphatics are the central lacteals in the intestinal villus in the mucosa. The central lacteals are located close (~50 μm) to the epithelium, which facilitates clearance of absorbed water and dietary lipids. These lacteals begin as blind-ended saccules that fuse with the submucosal lymphatic networks and penetrate into the muscular layer [147]. The lymphatics in the submucosa and muscular layers deliver their contents to collecting lymphatics that transfer lymph to mesenteric lymphatics.

Although unidirectional valves have been described between mucosal and submucosal lymphatic networks [148], these structures may not be extremely important physiologically to the overall conduction of lymph from the small intestine, since tracers injected into the submucosal layer can be seen to reflux into the mucosal layers. Because such valves are not locally preventing retrograde flow, a layer of valve-free lymphatics has been reported between the circular and longitudinal external muscular layers, which may exploit the peristaltic massage by these layers to accomplish fluid transportation without valve-bounded segments.

### 2.2.7 Lymphatics within the Large Intestine

Whereas the small intestine is densely supplied with lacteals, the large intestine lacks lymphatic lacteals but is still well supplied with initial lymphatics and collectors. Also, although initial lymphatics in the small intestine are located just below the mucosal surface, lymphatics in the colon lie much deeper in the mucosa (~300–400 microns under the mucosal epithelia). These lymphatics therefore have a more extensive network in the submucosal layer, which accomplishes similar fluid absorption [149]. Lymphatic capillaries in the large intestine also run vertically next to crypts of Lieberkuhn (Paneth cells), which are important in mucosal defense and immunity. The submucosa contains the highest lymphatic network density in the large intestine and has active unidirectional valves which run alongside blood vessels supplying the tissues.

It should also be noted that lymphatic structural arrangements vary in different mammals depending on size, diet, and nutritional strategy. For example, rabbits have blood capillaries just below the mucosal layer with horizontal lymphatic capillary networks found closer to the muscularis mucosa [150]. In dogs, networks of "shallow" lymphatic channels are arranged in a dual-layer; one just under the capillary networks below the mucosa, and a deeper layer of lymphatics more superficial than the muscularis mucosa [151]. In cats and dogs, lymphatic

vessels that traverse the muscularis layer have valves that may limit lymph reflux during contraction and may participate in lymph propulsion.

## 2.3 LYMPHATICS AND PROTEIN-LOSING ENTEROPATHIES

Lymphatic failure leading to lymphatic congestion causes increased interstitial and lymphatic pressures, and in the intestine, provoke inflammatory and ulcerative diseases. Two important types of protein-losing enteropathies, congenital/primary intestinal lymphangiectasia (also known as "PIL" or Waldmann's disease) and intestinal lymphatic hypoplasia (ILH), show some clinical similarities. PIL ("Waldmann's disease") [152] is an early onset disease in which the lymphatics of the small intestine become distended and leak fluid and protein. The causes for PIL are not well understood but appear to reflect incorrectly formed lymph vessels. PIL can be diagnosed by small intestine biopsies to reveal lymphatic dilation.

In additional to showing protein-losing enteropathy with nausea, abdominal pain, diarrhea/steatorrhea (fatty stools) [153], lymphedema in PIL may also develop in lymphatics at sites distant to the GI tract [154]. For example, PIL can also cause bilateral edema of lower limbs. The lymphangiectasia (dilation of lymphatics) in PIL shows up as blocked and distended lacteals in the small intestine, which causes lymph to leak from the gut back into the intestinal lumen, leading to poor absorption of protein and fats. This leak not only prevents absorption but also leads to a loss of serum albumin and immunoglobulins in PIL. Alpha1-antitrypsin that is lost from the blood into the feces has been used as an indicator of protein loss from the gastrointestinal tract, and because Alpha1-antitrypsin is an endogenous inhibitor of proteases (e.g., elastase and is anti-inflammatory and anti-microbial) that leads to increased susceptibility to infection [155]. This "low protein edema" is one reason why PIL is a type of "Idiopathic hypoproteinemia." The loss of Alpha1-antitrypsin is also seen in Crohn's disease [155].

The mass loss in PIL can be large, and depending on the fluid loss, lymphocytes can also be shed from the blood circulation, producing lymphopenia and immunodeficiency. PIL may exhibit alterations in VEGFR-3/LYVE-1 expressing vessels with high numbers of vessels in the intestinal mucosa, but fewer vessels in the deeper mucosa; overall levels of VEGF-C and D were also reduced. Therefore PIL may be due to insufficient signaling through VEGFR-3 based on low levels of its ligands and thus failing to maintain normal lymphatic homeostasis. In PIL, intestinal lymphatics may have reduced expression of the lymphatic patterning genes FOXC2 and SOX18 [156] but have normal Prox1. Waldmann's disease is treated by low-fat,

high protein diet with albumin supplementation, which helps to limit lymphatic dilation and hypoproteinemia, and surgery as a last option.

## 2.4 OTHER FORMS OF PROTEIN-LOSING ENTEROPATHIES

Lymphatic mediated protein enteropathy has also been seen in lupus and other collagen vascular diseases like scleroderma, which can provoke malabsorption due to fibrosis of lymphatics. Whipple's disease, types of obstructive lymphomas, sclerosing mesenteritis (panniculitis), mesenteric sarcoidosis and mesenteric tuberculosis can also include PLE. Lymphangioleiomyomatosis, a rare smooth muscle hyper-proliferative condition which creates obstructive pulmonary vascular nodules, also show chylous disturbances and can produce protein-losing enteropathy [157].

Forms of venous obstruction like right heart failure, constrictive pericarditis [158], familial cardiomyopathy [159], interatrial septal defects [158], Lutembacher's syndrome (septal defect with mitral stenosis) [160], and pulmonary stenosis [161] can all provoke protein-losing enteropathies by distending lymphatics, leading to a loss of lymph protein as well as lymphocytes.

## 2.5 INTESTINAL LYMPHATIC HYPOPLASIA

Intestinal lymphatic hypoplasia is a protein-losing enteropathy often detected from birth [162] that exhibits fewer podoplanin$^+$ (D2-40$^+$) vessels, which impairs gut fluid and fat absorption, also leading to the failure to retain protein [163].

### 2.5.1 Liver

A major portion of total lymph is formed in the liver, with hepatic lymph accounting for as much as 50% of the total lymph flow. Most hepatic lymph originates at the hepatic sinusoids. Lymph is drained by two major pathways in the liver: the right lobar path is the main pathway, and the left lobar pathway is an accessory lobar path [164].

Lymph formed in the hepatic sinusoids enters the space of Disse and collects in lymphatic capillaries associated with portal triads. Once the lymph reaches the space of Disse, it then drains into deep lymphatics associated with portal triads. The initial lymphatic capillaries of the liver found in interlobular connective tissue are parallel to blood vessels. The lymphatic

system in the liver is an integral part of the hepatic microcirculation and is clearly associated with arterial branches and, to lesser extent, with branches of hepatic veins. The fibrous liver capsule contains superficial lymphatic vessels. Endothelial cells of the liver sinusoids are fenestrated and lack basal lamina, which allows bulk flow of both fluid and proteins, allowing free passage to the space of Disse (located between the endothelium and hepatocytes), which results in the formation of hepatic lymph. LYVE-1 is expressed both in the sinusoidal as well as lymphatic endothelial cells [165], and reelin is found in the "Ito" cells of the liver [166].

It has been demonstrated that the only "true" lymphatics of the hepatic acinus are located in the portal tracts [167]. Small lymphatic vessels with diameter less than 25 μm form periportal venular network and are found to be associated with the limiting plate of hepatocytes. Interstitial fluid from the liver sinusoids flows along the perisinusoidal space, or space of Disse, which is contiguous with the interstitial space of the portal tracts at the origin of the sinusoids. This fluid then travels to portal tracts near the portal venules and enters the small lymphatics that are adjacent to the portal venules, which merge with larger vessels near hepatic arterioles and bile ducts. The portal tract lymphatics of the liver drain from the interstitial space, continuous with the space of Disse where sinusoids emerge from the portal tract.

Restriction to hepatic venous outflow is seen in veno-occlusive disease, Budd-Chiari syndrome, and in congestive hepatopathy. Even minor opposition to hepatic venous outflow will increase lymph flow. Because sinusoidal endothelial permeability is high, an elevation in liver sinusoidal pressure beyond the normal pressure of 3 to 5 mm Hg will produce an increase in lymph formation. Greatly increased sinusoidal pressures cause lymph to "weep" from surface of the liver and this fluid will accumulate in the abdomen as "ascites fluid" [168]. Increased liverlymph flow can raise pressure in the cisterna chyli, and restrict outflow of intestinal lymph to intensify intestinal edema [169], linking hepatic lymph management to gut inflammation. During portal hypertension or cirrhosis, the increased flow of fluid and solutes from the gut lumen into lymph bypasses the liver and delivers several inflammatory mediators to lymph, often with toxic consequences.

Whenever hepatic venous outflow is blocked, producing portal hypertension (as in cirrhosis/end-stage liver disease), increased hepatic lymph formation will dilate the peri-sinusoidal space of Disse. This is conspicuous in acute Budd-Chiari syndrome where blocked hepatic venous outflow results from thrombosis.

The lymphatic endothelial determinant Prox-1 also plays a roles in liver development by controlling fetal hepatocyte migration [170]. The livers of Prox-1-deficient mice have smaller amounts of hepatocytes. Molecularly, this phenomenon reflects Prox-1 suppression of E-cadherin, which prevents epithelial-mesenchymal transition [171]. In adults, Prox-1 also maintains liver homeostasis in and is needed for liver remodeling.

## 2.6 CIRRHOSIS

Cirrhosis is a late-stage fibrosis of the liver which can appear due to many causes including hepatitis or alcohol consumption. Portal venous obstruction can produce portal hypertension and greatly increase liver lymph flow. In cirrhosis, flow of the lymph can increase up to ten times normal [172], which can also dilate the thoracic duct several times its normal size. The hepatic lymphatic overload in cirrhosis reflects sinusoidal hypertension, which increases lymph formation. Cirrhosis also usually increases the numbers of lymphatic vessels [165], which may represent a physiological adaptation. During portal hypertension or cirrhosis, the increased flow of fluid and solutes from the gut lumen to lymph allows several classes of bioactive mediators to enter the lymph, bypassing the liver where they might normally be degraded, sometimes with morbid consequences.

## 2.7 ROLES OF LYMPHATICS IN THE DEVELOPMENT OF ASCITES

Ascites, the development of abdominal swelling as a result of accumulated intraperitoneal fluid, is tightly linked to lymphatic drainage. Under normal conditions, peritoneal fluid is formed at a low rate and lymphatics within the diaphragm are able to clear fluid out of the peritoneal cavity. Cells and particles as large as India ink are able to enter diaphragm lymphatics, which communicate with peritoneal and pleural mesothelial lining cells that are structurally and developmentally connected with lymphatics. Restricted outflow obstruction or hypertension in the liver or intestine or blockage of peritoneal drainage will increase the burden of ascites fluid in the abdomen. Defects in lymphatic development and/or structure can also provoke “chylous” ascites which appears white, reflecting the fat content [173].

### 2.7.1 Gallbladder

Human gall bladder lymphatics consists of three sets of lymphatic networks: muscular, perimuscular, and subserosal. The gallbladder also shares lymphatic networks with bile ducts. These networks, referred to as the sub-mucosal and sub-serous lymphatic plexuses, adjoin the gallbladder and the bile ducts and drain into the hepatic nodes, specifically the cystic node and the epiploic foramen. Lymph that is collected here then proceeds to intestinal lymphatic trunks, then finally to the thoracic duct. Lymphatic vessels are indeed present in all these regions;

however, the highest lymphatic vessel density (LVD) is in the perimuscular, followed by the muscular, and the lowest in the mucosal layers. The high LVD in the perimuscular layer may promote tumor metastasis; in fact, patients with tumors invading this layer are found to have poorer prognosis compared to patients with tumor lymphatics restricted to the superficial layers [174]. Gallbladder lymphatics are connected with lymphatics of the liver and the pancreas, and this interconnection may have pathophysiological significance: for example, experimental inflammation of the gallbladder resulted in inflammatory changes in the pancreas that likely involved, and was promoted by, lymphatic transport [175].

### 2.7.2 Pancreas

The flow of lymph in the pancreas is much lower than that of the intestine. Chronic pancreatitis can destroy pancreatic lymphatic channels under the capsule [176], indicating that disturbances in pancreatic lymphatic outflow might intensify forms of pancreatitis, whereas increased lymphatic flow may protect the pancreas from injury. The blockade or ligation of pancreatic lymphatic outflow vessels produces an intense pancreatitis via local release of toxic proteolytic enzymes. The release of these cytotoxic enzymes into "shock" lymph (see section below on **Shock, Lymph and the GI System**) is a significant risk for disease complications where obstruction is present.

In addition to functioning as a regulator or lymphatics, Prox-1 also functions as a critical governor of pancreas "bud" development [177]. In the absence of Prox-1, mice show disturbances in pancreatic epithelial organization and the size of the pancreas is reduced, but endocrine cell development is not impaired. However, by day 13.5, Prox-1 deficient mice have reduced numbers of pancreas islet precursors, so that relatively fewer cells are available to subsequently mature into the pancreas.

Prox-1 is abundantly expressed in the adult pancreas [178], linking adipogenesis, insulin action, and obesity. Although Prox-1 is needed developmentally, conditional elimination of Prox-1 in a "mature" pancreas shows that Prox-1 maintains pancreatic integrity and when suppressed, Prox-1 loss causes exocrine cell apoptosis, inflammation, and impaired function of the pancreas.

In non-obese diabetes, lymphatic endothelial cells in the pancreas can change their phenotype, losing their expression of 5'-nucleotidase and increasing expression of CCL21 (secondary lymphoid chemokine) in an age-dependent fashion, supporting immune cells' infiltration of these tissues [179]. This indicates that factors which control pancreatic and lymphatic health may be closely linked and dysregulated in diabetes and obesity.

The pancreas has an extensive lymphatic network, most of which is found within the interlobular septae that divide the pancreatic lobes and nodes, and arms of these lymphatics penetrate into the nodes. Interlobular lymphatics are not dense, and in other regions of the pancreas, lymphatics follow blood vessels. Lymphatics in the pancreas do not penetrate to the islets of Langerhans [180].

Both endocrine and exocrine products of the pancreas can be found in pancreatic lymph, but this does not seem to represent a significant pathway for hormone secretion under normal conditions since lymph flow is so slow (0.009 ml/min/100 g). Therefore pancreatic lymphatics serve essentially a fluid homeostatic function by regulating interstitial balance and clearing some pancreas secretions which reach the interstitium. If the pancreas lymphatic drainage function is inadequate because of blocked outflow in response to injury or inflammation, its secreted exocrine products like proteases (e.g., trypsin, chymotrypsin, ribonucleases, deoxyribonuclease, gelatinase, elastase) may accumulate in the interstitium, where they can rapidly damage tissue to cause injury and edema. Such injury can rapidly intensify pancreatic injury, leading to vicious cycles of re-injury and fibrosis which may increase the severity of pancreatitis.

Pancreatitis exhibits evidence of injury to both blood vascular and lymphatic networks, producing both thrombosis and hemorrhage, and can also elicit profound hypotension. Pancreas lymph flow increases during active pancreatitis [181], with increased levels of amylase and lipases in thoracic lymph. The pathophysiology of obstructive pancreatitis has been described where the interruption of normal lymph flow out of the pancreas leads to significant local tissue destruction. Similarly the downstream failure to clear lymph coming from the pancreas via the cysterna chyli provokes intense inflammation [182].

Induced obstruction of pancreas lymphatic outflow is lethal, producing fatal necrotic pancreatitis through pancreas "autodigestion," wherein the release of a cocktail of highly toxic mediators can provoke an intense form of shock [183]. Insulitis, an islet of Langerhans inflammation of the pancreatic β-cells (and sometimes exocrine tissues), is mediated by the influx of T- and B- lymphocytes, macrophages, and dendritic cells to provoke clinical diabetes. In experimental insulitis, pancreatic lymphatic endothelium shed 5'-nucleotidase/CD73 and upregulated secondary lymphoid tissue chemokine (SLC/CCL21), which decreases adenosine to increase permeability and increases lymphatic retention of lymphocytes and dendritic cells [179]. In acute pancreatitis without outflow obstruction, a large collection of toxic species including enzymes (trypsin, amylase and lipase) as well as cytokines (tumor necrosis factor-α, interleukin-1β, interleukin-6) are present in pancreatic lymph that is delivered to the thoracic duct [184, 185]. Lymphatic cannulation, which diverts these products from the circulation,

prevents the development of respiratory distress injury which would normally accompany this clinical syndrome. Stimulation of the pancreas by cholinergics like pilocarpine triggers the release of pancreatic amylase, lipases, and proteases which can appear in lymphatics [186]; excess secretion into lymph can trigger pancreatitis.

Although Prox1 can suppress E-cadherin, which is a tumor suppressor gene, decreased Prox-1 expression may also contribute to loss of differentiated (such as epithelial-like) phenotype and progression to transformation with poorer outcomes. Prox-1 (and beta-catenin) expression in the pancreas both appear to be absent during malignant transformation and are negative outcome indicators. In this sense, loss of one functional Prox1 allele increases the trend for tumor development in the pancreas [187].

### 2.7.3 Renal (Kidney) Lymphatics

Renal lymphatics are found in the cortex of the kidney (where there is a subcapsular lymphatic network) and in the cortical medulla region, although lymphatics appear to be sparse in the medulla. Lymphatics are abundant among arteries and veins (particularly the interlobular and arcuate), but very few lymphatics near the glomeruli or cortical tubules. Renal lymph drains from the hilum (urinary duct) through several small hilar lymphatics. Because of the location of the lymphatics in the kidney, renal lymph is essentially an ultrafiltrate of glomerular capillaries within the cortex. Renal lymph has a composition similar to lymph formed in other anatomic locations but has a lower creatinine content and a higher proportion of albumin than plasma. Renin is also higher in lymph than in blood (about 8-times more based on release by the juxtaglomerular cells). Renal lymph flow in sheep varies between 0.5–3.0 ml per hr.

In end-stage renal disease, lymphatic density may be increased in response to the ongoing inflammation and tissue injury in this condition [188]. Renal cell carcinoma often shows increased numbers of lymphatics at the tumor margin, but these do not enter the tumor possibly due the high interstitial fluid pressures within the tumor, compared to the more extensive blood vessel invasion of the tumor beyond the tumor margin.

### 2.7.4 Myocardial (Heart) Lymphatics

The heart is very well supplied with abundant lymphatics and does not appear to tolerate accumulations of interstitial fluid. The myocardium seems to be able to adapt to rapidly changing rates of interstitial fluid evolution under conditions of exercise, ischemia, and other types of cardiac stress. Cardiac lymph flow is prodigious considering its tissue mass. Miller (year/

reference?) has estimated a basal (at rest) lymph flow of 3.2 mls per hour (in dogs). This rate of lymph formation can be increased eight times during stress.

The origin of motive force underlying lymph propulsion in the heart has traditionally been assumed to be almost entirely produced by myocardial compressions resulting from normal cardiac contractions, which would force lymph forward. This is not entirely clear, however, and lymphatic vessels have muscular investment and may establish tone. Phasic contractions of cardiac lymphatics appear to exist and may play a role in cardiac lymph transport (*Albert Miller, personal communication*). Regardless of the source of the compressive forces driving lymph movement, there are abundant lymphangions present in the epicardium (revealed by kB-lacZ staining) [30]. The heart can also adaptively reorganize and remodel its lymphatic supply via lymphangiogenesis, as has been seen in several other organs such as the intestine. It is not clear whether such remodeling is beneficial by way of relieving fluid burden or whether the expanded lymphatic network increases immune-mediated stress responsiveness. With respect to fluid balance, the importance of cardiac lymphatics is quite clear in that experimental obliteration or obstruction of cardiac lymphatics causes a devastating loss of cardiac contractility, as well as pacemaker signal origination and coordination. In as little as 48 hours following cardiac lymphatic obstruction, electrocardiographic disturbances which bear a resemblance to myocardial infarction can occur. These issues can involve alterations in impulse formation (pacemaker) and conduction defects such as atrioventricular node block. Similarly, forms of myocarditis also reveal functional disturbances which suggest histological evidence of lymphatic obstruction and dilation.

## 2.8 SKELETAL MUSCLE LYMPHATICS

Lymphatics are also critically important for the appropriate functioning of skeletal muscle. In skeletal muscle, terminal lymphatics develop next to postcapillary venules and pass into the perimysium where they join larger lymphatic trunks that lie near paired arterioles and venules. Lymphatic vessels which are 20–200um in diameter (in rats) and are found within the muscle itself do not have muscular walls and consequently depend upon contraction to provide the motive force for moving lymph back towards the bloodstream [189]. Although muscular lymphatics are absent within the muscle itself, spontaneously contracting lymphatics are found on the surface of skeletal muscles and serve an accessory role in lymph propulsion. These lymphatics have been reported to have bi-leaflet valves [190]. The ability of skeletal muscle contraction to influence lymphatic propulsion appears to reflect the location, alignment, and depth of lymphatics relative to the muscle fibers and likely the localized coordination of muscular contractions

[191]. Lymphatics are also found around larger arteries in skeletal muscle where rhythmic arterial pressure pulsations periodically compress the neighboring lymphatics and further support central lymph transport.

## 2.9 LYMPHATICS AS A HORMONE TRANSPORT SYSTEM?

Lymphatics act a parallel transport system with the cardiovascular system, and lymphatics can transfer hormones and autacoids which reach or accumulate in the interstitium of the anatomical locations where they are formed. Because materials reaching the interstitium may include mediators, hormones, and autocoids released by tissues and by immune cells reaching these tissues, lymph may therefore represent an under-appreciated type of endocrine transport conduit.

For example, insulin has been measured in thoracic duct lymph [152], and insulin transported in visceral lymph might bypass the liver to retain more than half of the insulin which is destroyed via "first-pass" portal venous flow [192]. It has been suggested that the insulin found in lymph appears to reflect filtration from blood, rather than direct release into lymph. However, delivery of intramuscularly delivered insulin (and other materials) may quantitatively arrive in the lymph following essentially an interstitial administration by intramuscular routing [180]. It was reported that normal pancreatic exocrine or endocrine insulin secretion into the thoracic duct is not a physiologically important route, however, this pathway can become relevant in the setting of acute or chronic pancreatitis.

Lymphatics can also participate in the catabolism of catecholamines, which reach and are collected by the interstitium, as well as histamine and xenobiotic amines which are processed into pharmacologically-inactive aldehydes. Perhaps one of the most important materials carried by lymphatics are lipids, and consumption of a fat-laden meal transfers lipids and cholesterol from lymph into the blood. Several enzymes have been described in lymph which might catabolize several classes of bioactive mediators, including aldolase, alkaline and acid phosphatases, lactate dehydrogenase, diamine oxidase, and amylase.

CHAPTER 3

# Lymphatics in Pathophysiological States

## 3.1 LYMPHATIC DISTURBANCES INTENSIFYING CHRONIC INFLAMMATORY DISEASE

Heritable defects in lymphatic patterning and structure may affect lymphatic pump function and can lead to lymphostasis and lymphatic obstruction. Inherited defects in lymphatics (or prevention of lymphatic remodeling, e.g., radiation) may intensify several forms of chronic inflammation, worsening pathology and exacerbating tissue injury.

The rate of lymph flow is influenced by a large variety of environmental and pharmacological factors. For example, the degree of hydration controls the amount of fluid present within the interstitial compartment. Other environmental influences that can affect lymph flow include forces external to the lymphatics which may compress fluid in the lymphatic interior, like the massaging forces created during normal gut peristalsis (extrinsic forces). Intrinsic compressive forces are also generated during normal lymphatic phasic contractility. Additionally, the state of "tone" of the lymphatic vasculature may also influence the ability of these vessels to transport fluid by regulating the diameter of these vessels, which may influence the forcefulness with which these vessels are able to contract. If vessel tone is low, for example, vessels may be dilated and unable to contract as rapidly or strongly as when they are partially contracted. It is also possible that the extent of vessel dilation may also influence the ability of valve leaflets to function as well as it would in more fully dilated situations.

As an example, in the gastrointestinal tract, lymphatics normally help to maintain interstitial fluid balance by continuously clearing fluid which enters the submucosal space. During forms of chronic inflammation (e.g., in inflammatory bowel disease, especially Crohn's disease but also ulcerative colitis), lymphatic clearance is often impaired by lymphangitis and is insufficient to prevent interstitial fluid accumulation producing dilated lymph vessels and tissue edema. This type of lymphatic insufficiency-mediated elevation of interstitial fluid pressures can result in an increased distance between blood vessels and parenchymal cells, leading to a failure to deliver nutrients and oxygen and collect waste. The increased pressure also opposes blood perfusion, which can interfere with the delivery of immune cells to the mucosa. Once these immune cells

reach the interstitium, they may be delayed in progressing out of the tissues via lymphatics, and the prolonged residence times of inflammatory immune cells within the gut may exacerbate the immune cell-mediated tissue injury that accompanies forms of chronic inflammation in the gut. Additionally, if the uptake and transfer of "normal" gut commensal bacterial antigens by mucosal macrophages is impaired by edema and lymphostasis, impaired transport of immune cells in gut lymphatics may trigger an inappropriate adaptive immune response towards normal gut flora which can contribute to the pathogenesis of IBD-associated inflammation.

## 3.2 LYMPHEDEMA

Lymphedema is a condition in which swelling occurs in the arms or legs, usually resulting from a blockage in the lymphatic system.

Primary vs. Secondary Lymphedema: We recognize two classes of lymphedema: 1) congenital (hereditary) lymphedema, arising from genetic disturbances that produce structural malformations or errors in lymphatic patterning or programming, and 2) secondary forms of lymphedema, which are caused by either medically or infection (usually filarial)-induced obstruction. Forms of primary, congenital lymphedema are also defined by the age of onset: hereditary lymphedema type I appears before age 35; type II appears after age 35.

Primary causes of lymphedema include genetic problems which lead to the failure of lymphatics to develop properly, show aberrant patterning, or fail to appropriately remodel after stress or injury. Secondary causes of lymphedema include cancer therapy, especially radiation therapy (which damages lymphatics) and prophylactic removal of lymph nodes during tumor resection; infections which can damage lymphatics; rheumatoid arthritis; dermatitis, and eczema, which can cause lymphedema as well as skin burns or deeper skin injuries.

The swelling of limbs, perhaps the most common feature of lymphedema, can range from mild to severe and is associated with progressive fibrosis. Pain is usually associated with more severe staging, as are problems with mobility of the affected limbs. Radiation itself can prevent normal lymphatic regrowth, potentially contributing to lymphedema, and anti-breast tumor agents like Docetaxel can also increase the risk of developing lymphedema [246].

## 3.3 DIAGNOSIS OF LYMPHEDEMA

The diagnosis of hereditary lymphedema can be confirmed by several imaging techniques which include lymphoscintigraphy, radio-lymphography, ultrasound, and magnetic resonance imaging

(MRI). Lymphoscintigraphy measures the relative rate of clearance of radioactive labeled tracers injected into the skin into lymph nodes, which is reduced in forms of lymphedema. Individuals suffering with forms of lymphedema (either active or congenital forms of lymphedema) will fail to actively clear these lymphatic tracers from the site of injection. MRI imaging can reveal consequences of lymphedema, which include tissue edema, lymphocoeles (accumulated pockets of lymphatic fluid), and fibrotic changes which often accompany lymphedema. Ultrasound is also commonly used to gauge disease severity, such as thickness of skin and subcutaneous tissues.

## 3.4 CLINICAL COMPLICATIONS OF LYMPHEDEMA

Because elevated tissue pressures in lymphedema can impair blood perfusion and drainage, it may be difficult to supply tissues with immune cells. Dermal lymphostasis due to lymphedema dramatically increases the risk for the development of infection, ranging from superficial (e.g., erisepylas, an infection of the upper skin layers and their lymphatics) to subcutaneous and deep skin infections (such as *cellulitis*, often caused by *Staphylococci* and *Streptococci*). Lymphedema also increases the risk for fungal infections. Although not normally a significant health risk, conditions like Athlete's foot infections (*Tinea pedis*) may represent a more serious situation in the setting of lymphedema, as it may become a nidus for more extensive and serious secondary (bacterial) infections such as *Staphylococcus*.

In addition to skin infections, lymphedema can also lead to more serious disseminated systemic infections, and persistent cellulitis can require disinfection that can cause necrosis and intense scarring and disfigurement. Other complications of lymphedema can include deep venous thrombosis, localized or systemic sepsis, and gangrene (which can occur in severe cases, requiring debridement or amputation). Skin issues in more long-standing lymphedema can include dry and splitting skin; the underlying skin can also become thicker and harden, producing a cobblestone appearance (*hyperkeratosis*); reddened raised bumps can also appear (a form of *papillomatosis*.)

Lymphangitis (inflammation of the lymphatics) often results from Streptococcal infections, and lymphostasis, the obstruction of lymphatics and failure to clear interstitial fluid, produces intense edema, and represents a serious complications of lymphedema. Lymphorrea (abnormal lymph flow out of damaged lymphatics) is also often encountered.

## 3.5 LYMPHEDEMA DISTICHIASIS

Lymphedema distichiasis (LD) was originally described by Falls and Kertesz in 1964 as "Falls-Kertesz syndrome," but it is now known as Lymphedema distichiasis syndrome (LDS). LDS is

diagnosed by physical exam, family history and genetic screening. Its severity can be confirmed by lymphoscintigraphy. LDS is a rare autosomal dominant disorder which causes lymphatic disturbances of variable severity in legs which show edema due to accumulation of interstitial fluid, and many organ systems may be affected. The term "distichiasis" refers to the appearance of an extra set of eyelashes that grow at the rear border of the eyelid. In individuals afflicted with LDS, lymphedema often begins during puberty and most often affects lower limbs.

LDS appears to be produced by at least 30 autosomal dominant pathogenic mutations in the FOXC2 gene which in humans is located on chromosome 16 [193]. Mice with one normal FOXC2 gene copy (haploinsufficiency) still show disturbances in lymphatic drainage similar to those seen in humans with LDS [194], which include lymphedema in the extremities and lymph reflux (retrograde movement) within the gastrointestinal tract [195]. However, significant gastrointestinal symptoms are not a common feature of LDS in humans.

Additional features of human LDS can include developmental problems including widening of the spinal canal, visual problems (including corneal scarring and astigmatism), ptosis (lid drop), cardiac abnormalities, and cleft palate. Lymphedema in LDS appears to reflect hypo-functioning lymphatic valves and the lymphatic vessels also have a higher than normal muscular investment [68], reflecting increased PDGF-mediated recruitment of mural cells. Individuals with LDS may also show expansion and proliferation of lymph nodes.

In LDS, lymphedema usually starts around puberty and affects the legs and thighs, often affecting one limb more than another. Other independent variables lead to differences in severity between affected individuals from different families. LDS is equally distributed in males and females, but shows more severe disease in males, who show earlier appearance of disease and more frequent complications [196]. LD usually appears in later childhood or at puberty, most often producing lower limb lymphedema and can exhibit additional eyelashes from the inner lining, up to a double row (distichiasis) which confirms the diagnosis. Venous insufficiency also occurs in half of LDS sufferers; LDS can also exhibit spinal problems, corneal and visual problems, ptosis (eyelid drooping) and alterations in craniofacial and cardiac structure. Figueroa Syndrome is another autosomal dominant form of an FOXC2 mediated hereditary lymphedema reported by Figureroa (1983), FS also displays facial disturbances (cleft palate).

## 3.6 NONNE-MILROY-MEIGE SYNDROME

Meige's lymphedema (hereditary lymphedema type II) is the most common form of lymphedema, affecting approximately 1/100,000, with a gender tendency for females to show the

disease 3 times as often as males. Meige's lymphedema begins at puberty and is named for Dr. Henri Meige (who first described hereditary lymphedema in 1891). Meige's syndrome accounts for approximately 80% of hereditary lymphedema. As a congenital form of lymphedema, Meige's lymphedema is present from conception, but often does not become apparent until puberty when the swelling frequently starts in the feet and ankles and then develops in the legs up to the knees, although other limbs, skin, and organs can also be affected. Meige's lymphedema usually appears by age 35 years and shows hypoplasia of peripheral lymphatics and dilation of lymphatic networks. Yellow nails can also be present due to ?????. Meige's disease appears to be caused by mutations in FOXC2. Diagnosis is made by lymphoscintigraphy, which reveals lymphatic hypoplasia and transport failure and by genetic screening.

### 3.6.1 Nonne-Milroy Disease and Meige's Syndrome

Milroy's disease is a hereditary form of lymphedema most commonly affecting the legs which is often present at or near birth. It is frequently bilateral and in males can show scrotal lymphedema (hydrocoele). NMD shows fibrosis of limbs and erysipelas (superficial dermal infections) to cellulitis (deeper skin infections). Nonne-Milroy-Meige syndrome now appears to be two distinct conditions: Nonne-Milroy (or Milroy's disease/hereditary Lymphedema type I) and Meige's syndrome (a hereditary lymphedema type II), also called "*familial lymphedema praecox*."

Milroy's disease (NMD) [197] is named for William Milroy, who documented cases in 1892; Rudolf Virchow, famous as the champion of public health, also described Milroy's disease. Milroy's historical family report on this condition, described 22 affected individuals over 6 generations initially describing this condition starting in 1728. As a form of congenital lymphedema, NMD generally appears early in life, mainly females, presenting as a unilateral lower limb edema that can develop as bilateral edema. NMD is autosomal dominant disorder, and is the least common form of heritable lymphedema, making up only 2% of congenital forms of lymphedema. Issues in NMD are created by many different inactivating defects in VEGFR-3 signaling. These have been mapped to chromosome 5q (5q34–q35 region). Gordon et al. (2013) described 58 different variants in VEGFR-3 [198].

Congenital mutations in VEGFR-3 can also lead to defects in lymphatic development and maturation with lymphatic disturbances [36]. Symptoms of NMD include steatorrhea (fatty stool, reflecting the failure of intestinal lymphatics to adequately clear dietary fats/cholesterol) and chylous ascites is also apparent. NMD is also a type of protein-losing enteropathy, and the lymphatic failure in NMD also creates an inability to retain and re-circulate lymphocytes. Lymphopenia is apparent in NMD and cell-mediated immunity is diminished in these individuals.

Currently, Meige's syndrome appears to be distinct from NMD. Rezaie et al. suggest separating these based on VEGFR-3 vs FOXC2 mutations, where FOXC2 defects should reflect lymphedema distichiasis but not MDS [199]. NMD is characterized by fibrosis of limbs, with erysipelas (superficial skin infection) and cellultis (deeper skin infections).

NMD appears to be caused by defects in VEGFR-3, leading to the failure to develop normal lymphatics. Because VEGFR-3 is also involved in venous development, venous disturbances may also be present. As an autosomal dominant condition, inheritance of one defective copy of the FLT4 gene (VEGFR-3) (found on chromosome 5) can produce this disease. Therapy for Milroy's disease is symptomatic and decongestive (see section below on **Treatments for Lymphedema**).

## 3.7 HENNEKAM'S SYNDROME

Hennekam's syndrome (HS) is a congenital autosomal recessive lymphedema described by Dutch physician Raoul Hennekam in 1989 [200]. This syndrome is produced by defects in CCBE1 (collagen and calcium binding EGF domains 1) (involving chromosome 15q and 18), which impairs the development of lymphangioblasts or protocadherin FAT4 (chromosome 4). Hennekam's syndrome is usually visible at or before birth, which usually produces a severe form of prenatal lymphedema (or "hydrops fetalis"). Infants afflicted with Hennekams' syndrome may have microcephaly, facial lymphedema, edema of the genitalia, cranial and facial abnormalities with facial flattening, edema of the eyelids, and hypertelorism (widely-space eyes). The size of the ears and mouth can also be reduced, with overgrowth of the gums. In the intestine, such distension can lead to tearing of the lymphatics and poor resorption of dietary lipids, vitamins, and proteins, leading to lymph accumulation in the abdomen (chylous ascites). Lymphostasis in Hennekam's syndrome can also affect the lungs, pericardium, skin, and kidneys.

Hennekam's syndrome also shows lymphatic distension (lymphangiectasia) reflecting an inability to clear interstitial fluid, with its accumulation especially within the intestine. HS is also a type of protein-losing enteropathy which decreases levels of circulating plasma proteins, including immunoglobulins and lymphocytes, which may lead to immunosuppression.

## 3.8 SHARP-AAGENAES SYNDROME

Sharp-Aagenaes syndrome is a congenital form of lymphedema, named for Norwegian physician Øystein Aagenaes. Aagenaes syndrome is more common in Norway, followed by Europe and the USA. Half of all cases have been reported in Norway, with ~3% frequency in gene

alteration in southern Norway alone. Aagenaes syndrome is an autosomal recessive condition that has been mapped to chromosome 15q. Dominant forms of what appears to be Aagenaes syndrome has been reported in non-Norwegians but the genetic basis of these forms has not yet been identified. Aagenaes syndrome is a form of cholestasis within the liver ("cholestasis lymphedema syndrome") which also affects the lower torso [201]. This condition appears to be due to abnormal lymphovascular channels in the peribiliary tracts.

## 3.9 NOONAN SYNDROME

Noonan Syndrome (NS) is a congenital, autosomal dominant condition which is characterized by short stature, the presence of a webbed neck, cardiomyopathy, and lymphodysplasia (lymphatic dilation). NS pathology is similar to Turners syndrome (XO) but is not sex-linked. Although lymphodysplasia is present, severe lymphatic pathology appears not to be a major symptom of NS; infants can transiently show lymphedema at birth, but only 1.2% show limb lymphedema. Some NS patients will exhibit lymphangiectasia in the intestine [202]. NS appears to involve mutations in tyrosine phosphatase-N11, (SHP-2) which is found on chromosome 12q24.1. Mutations in KRAS and SOS1 have also been associated with the development of NS [203] which has been classed as a "RASopathy."

## 3.10 YELLOW NAIL SYNDROME

Yellow-nail syndrome (YNS) (also called "primary lymphedema associated with yellow, dystrophic nails and pleural effusion") is a relatively uncommon lymphatic condition caused by impaired lymphatic drainage due to hypoplastic lymphatic vessels. YNS produces a slow nail growth, pleural effusions, pulmonary congestion, and limb and peripheral lymphedema, with an onset around puberty. Intestinal lymphangiectasia and protein losing enteropathy have been reported in YNS [204]. YNS has been reported to run in families and has been described as an autosomal dominant condition, however, the underlying genetic basis of YNS is uncertain and may be triggered by diverse environmental exposures such as dental devices and drugs like penicillamine [205].

## 3.11 LYMPHANGIOMATOSIS

Lymphangiomatosis is a genetic condition in which diffuse lymphangiomas (lymphatic cysts) are present in many different organs, particularly the bones and lungs. Although not a form of

cancer, these lymphatics invade and press on tissues. Excess accumulation of lymph in the heart and lungs is extremely debilitating and can be lethal.

## 3.12 GORHAM-STOUT'S DISEASE AND LYMPHANGIOMATOSIS

Gorham-Stout's Disease (GSD) was first reported in 1838 as a disorder which shows osteolysis, the proliferation of lymphatic vessels, and appears to involve abnormal osteoclast activation. Because of this bone destruction, GSD is also known as "vanishing bone disease" and causes pain and swelling. GSD can affect individuals at any age and affect the skull and jaw, ribs, vertebral column, and pelvis. Because lymphangiomatosis is also characterized by extensive proliferation of lymphatic vessels (which can also frequently affect the bones and lungs), GSD and lymphangiomatosis are often considered along the same disease continuum.

## 3.13 KLIPPEL-TRENAUNAY SYNDROME

Klippel-Trenaunay (KT) is a condition that shows congenital port wine birthmarks, overgrowth of blood vessels, skin, muscle and bone tissue, and venous abnormalities. KT shows lymphatic varicosities, lymph node abnormalities, and lymphedema. The PIK3CA mutations associated with KT syndrome alter the p110α protein.

## 3.14 LYMPHANGIOLEIOMYOMATOSIS (LAM)

Lymphangioleiomyomatosis (LAM), a clinical condition that affects mostly females, is caused by mutations in the TSC1 or TSC2 genes, which code for the proteins hamartin and tuberin respectively and are still not well understood. LAM exists in two forms, one linked with tuberous sclerosis (TSC-LAM) and the other form is known as sporadic LAM. In LAM, tumor-like cells which bear these mutations release VEGF-D, the lymphangiogenic growth factor. VEGF-D causes inappropriate cystic lymphoproliferation in the lung with pulmonary fibrosis, increased breathing effort, chylothorax, and chylous ascites. TSC1/2 products appear to inhibit mitochondrial targets or rapamycin (MTOR) C1, and consequently rapamycin has been evaluated for therapy in LAM. Based on the role that the VEGF-D/VEGFR-3 axis appears to

play in LAM, VEGFR-3 inhibiting drugs like MAZ-51 may also have applications in LAM therapy.

## 3.15 KAPOSI'S SARCOMA

In AIDS-associated Kaposi's sarcoma (KS), decreased T-cell mediated immunity often leads to failure to suppress human herpes virus-8 (HHV8), which can cause $CD34^+$ endothelial cells to express VEGFR-3 and become "spindle cells" that are characteristic of KS lesions. HHV-8 infected spindle cells appear to have several characteristics of lymphatic endothelial cells (VEGFR-$3^+$, podoplanin) and are negative for blood endothelial markers (e.g., PAL-E). KS skin and oral lesions appear red to purple skin because of the fusion of blood and lymphatic vessels with the release of blood cells within the lesion interstitium.

## 3.16 CANCER AND LYMPHATICS

The so called "sentinel lymph nodes" and their downstream lymphatics can transfer metastasizing tumor cells from an initial tumor site to new locations, and represents a major complication of tumor management. Sentinel lymph nodes are the first single or group of nodes which are identified by injecting visible or radioactive tracers near tumors and examining transport of the tracer to these nodes. Following this procedure, biopsy of these nodes is performed to see if tracers (and thus possibly cancer cells) have had access to spread to these nodes, a procedure which is often used for tumor staging and determining courses of therapy [206].

In cancer, the proliferation of cells in lymphatics can "clog" lymphatic channels and lymph nodes to produce lymphostasis, lymph reflux, and transfer of cancer cells from the lymphatic to blood circulation and transfer of cells to the peritoneal cavity by lymphatic to venous communication [207].

## 3.17 LYMPHATIC DISTRESS FOLLOWING CANCER THERAPY

Lymphedema of the arms remains one of the most difficult, disfiguring, and complex complications of breast cancer surgery, which includes mastectomy and lumpectomy resections for cancer. Secondary "iatrogenic" lymphedema is a devastating complication which can follow breast

cancer surgery that can affect roughly 30% of patients who have undergone breast cancer surgery especially with lymph node removal, with risks especially elevated for those patients who had received both surgery plus radiation. The five-year incidence of lymphedema varies widely and is up to 42% [208]. This statistic depends however on which scale is used to estimate severity. For example, "subjective" evaluations (patient's own and observers' personal impressions) found only 14% lymphedema, whereas more objective measurements (which include physical parameters of limb dimensions that see an increase in post-surgical volume exceeding 200 mL) found lymphedema in 25.5% of patients.

The "Memorial Symptoms Assessment Scale" has been frequently used to quantify patient's subjective patient impressions of lymphedema and includes perceived fit of clothing, jewelry, visibility of knuckles, veins, skin and tissue "puffiness," skin indentation and rebound, pain perception, and development of firm or leathery skin [209]. Therefore, the detection of limb edema can vary by nearly two-fold based on which evaluation is used, and significant post-surgical edema may often by present but not recognized [210].

The risk of lymphedema development following lymph node removal during cancer surgery appears not to be related to age, menopausal status, handedness or tumor staging, but does seem to be clearly related to the combined stresses of surgical resection plus the addition of radioablative therapy. The incidence of lymphedema in patients receiving axillary (armpit) resection plus radiation was 38.3% compared to radiation alone (8.3%) or axial dissection alone (7.4%).

Lymphedema measurement is often complicated with volume measurement being preferable to changes in circumference [210]. When lymphedema does occur, in 80% of the cases, it appears within 2 years of surgery, with 89% of lymphedema cases showing up within 3 years [208]. By 3 years following surgery, 23% of women had clinically "mild" disease, 12% had moderate to severe, and 2% had "chronically moderate" to "severe" disease. 37% of "mild" cases required treatment, whereas 68% of the moderate to severe cases were treated more aggressively.

## 3.18 WHIPPLE'S DISEASE

Whipple's Disease (WD) is a rare gastrointestinal disease caused by the gram-positive intracellular bacterial pathogen *Tropheryma whipplei*, discovered by G.O. Whipple in 1907. WD is one more disease in which small intestinal absorption is impaired, reflecting the injury to the small intestinal lumen which causes malabsorption. This condition is also characterized by weight loss, chronic diarrhea, weakness, fever, lymphadenopathy, arthralgia (joint pain),

polyarthropathy (joint injury), fatty stools, macrophage infiltration into the lamina propria, and lymphopenia [211]. WD strikes mainly males and can be lethal if not treated. Several pro-inflammatory cytokines like interleukin-12, interferon-gamma, immunoglobulin-G2 and TGF-β are all reduced in WD, and CD11b+ leukocytes become depressed in WD. With regard to lymphatics, WD shows evidence of lymphatic blockade, with engorged and often "fatty" lymph nodes (lymphadenopathy, lymphadenitis) and swollen lymphatic vessels [212]. Whipple cells have been isolated from thoracic duct lymph [213] and the lymphatics are the main route for the dissemination of WD cells and infected immune cells from the GI tract into the circulation. A similar dissemination of tubercle bacilli in lymph is seen in tuberculous enteritis, where lymphatics are known to transport these pathogens [214]. The isolation of lymph via flow arrest (lymphostasis) and the decrease in lymph pumping in response to pathogens may be adaptive in achieving a restriction of this type of pathogen spreading.

## 3.19 SHOCK, LYMPH, AND THE GI SYSTEM

In addition to disseminating pathogens, lymphatics (particularly gastrointestinal lymphatics) play central roles in creating and dispersing toxic products created in the gut and pathogenic species which reach the interstitium as a result of hemorrhagic shock, traumatic injury, and intestinal ischemia-reperfusion [215]. By carrying an enormous array of toxic mediators generated by the gastrointestinal system into the main blood circulation, lymph can "trigger" a systemic "distant" organ injury often with lethal consequences. Several GI organs, especially the post-ischemic small intestine, create "toxic" or "shock" lymph which activates inflammatory cascades like activating leukocytes, mobilizing endothelial P-selectin and ICAM-1, activates coagulation, increases adhesiveness of microvessels, and perturbs cardiac performance [216, 217].

Non-lethal hemorrhage of the mesentery produces a "shock" lymph that activates neutrophils [218], increases vascular solute permeability, and can induce apoptosis/necrosis (whereas these responses are not produced by portal venous plasma). The list of toxic mediators present in "shock" lymph which cause injury and cell stress include complement [219] and pro-inflammatory cytokines (like tumor necrosis factor-α, interleukin-6, interleukin-1β and LPS) [220, 221]. In particular, interleukin-1β (produced in the gut and carried in lymph) mediates an intense and remote lung injury following intestinal ischemia-reperfusion [222]. Although relatively removed from the mesentery, the lung and heart are often critical remote organ injury targets affected by "shock" lymph returned to the circulation via the thoracic duct [215, 223].

## 3.20 SJÖGREN'S DISEASE

Sjögren's disease (SD) is an inappropriate immune activation whose defect is characterized by a failure to produce saliva and tears; this often occurs as a comorbidity with rheumatoid arthritis and lupus. Garcia-Carrasco et al. [224] describe a lymphocytic infiltration of the salivary and lacrimal glands and a benign lymphoproliferation in SD. This lymphocytic activation gives SD sufferers an increased lifetime risk for developing lymphomas [225], however links between lymphatic disturbances and lymphoma in SD are not well understood.

## 3.21 LYMPHATIC PATHOPHYSIOLOGY IN GI DISEASES

Increased lymphangiogenesis has long been recognized as an important consequence of several forms of inflammation [226]. Because lymphatics are known to have important roles in lipid absorption and immune surveillance, especially in the gut, disturbances in gastrointestinal lymphatics are increasingly being recognized as central contributors in forms of acute and chronic large and small intestinal inflammation, especially in the setting of Crohn's disease (CD) and ulcerative colitis (UC).

Although Crohn's disease and ulcerative colitis are clearly different types of inflammatory diseases affecting different regions and layer of the gastrointestinal tract, they both show significant remodeling and dysfunction in lymphatic structure and function which may reflect the profound inflammation found in both diseases that may induce lymphatic activation. In Crohn's disease, lymphangitis and lymphangiectasia are central characteristics of CD and Crohn originally described this condition as a form of lymphangitis; this is often marginalized in contemporary descriptions of the condition, particularly now that more powerful treatments may suppress gut inflammation in CD. Although ulcerative colitis shows inflammation in the upper, superficial layers of the intestine and submucosa and is largely isolated to the large bowel, Crohn's disease (CD) is full thickness (transmural) and can affect any regions of the gastrointestinal tract from the mouth to the anus. CD and UC can both show enlarged as well as greater numbers of lymphoid aggregates, whereas epithelioid granulomas are limited to CD.

Both CD and UC show evidence for increased abundance of podoplanin-positive lymphatic vessels within the intestine [227] during active periods of disease. Geleff examined the abundance and spatial localization of podoplanin-positive lymphatic vessels in surgical specimens to evaluate the expansion of lymphatics in both ulcerative colitis and Crohn's disease.

Lymphatics in the small intestine were found under the blood capillary networks below the luminal epithelium, whereas lymphatics in the large intestine were observed in the outer third of the mucosal layer. In both forms of inflammatory bowel disease, lymphatics were significantly increased in the lamina muscularis mucosae (the thin muscle layer beneath the lamina propria), the submucosal and subserosal layers, and similar levels of expansion were seen in Crohn's and ulcerative colitis.

Interestingly, they reported that lymphatic vessels were conspicuous where chronic inflammation was changing to fibrotic tissue, and these alterations persisted into "end-stage" disease. Such profound lymphatic growth in the gut during inflammation might be a physiological response to offset the lymphatic failure that occurs when these vessels are obstructed or when lymphangion pumping failure occurs during inflammation [13]. This type of expansion must be critical to survival insofar as inflammatory signals produced by lymphangitis [228] may trigger the formation of fistulae ("pipes"), abnormal channels that communicate contents from the gut to the skin and other compartments (bladder, vagina), which reflects inappropriate activation of adaptive remodeling responses.

## 3.22 MENETRIER'S DISEASE

Menetrier's disease, also known as "hypo-proteinemic hypertrophic gastropathy," is an uncommon, pre-cancerous type of gastric hyperplasia of the mucous and surface epithelium. Menetrier's disease shows increased numbers of folds in the stomach lining and thickening of the mucosa with abundant production of mucus and protein loss, particularly immunoglobulins as well as diarrhea, all of which reflect lymphatic disturbances. The hyperplastic development of the epithelial lining and edema of the upper layers of the lining are correlated with increased numbers of lymphatics, lymph nodes, and lymphatic dilation (*lymphangiectasia*) in Menetrier's disease [229]. Adult onset disease can progress to adenocarcinoma.

The form of Menetrier's disease seen in children often follows respiratory infections or infection with cytomegalovirus and is often acute (months long duration) and heals spontaneously. By comparison, the adult onset form is more chronic and may be activated by *Helicobacter pylori* infection and is mediated by TGF-α [230]. This form is treated using Cetuximab, an EGFR blocking biologic. Both pediatric and adult forms of Menetrier's are protein-losing gastropathies (where proteins are lost from the gastric lining).

## 3.23 HERITABLE RISK FOR DEVELOPING POST-SURGICAL LYMPHEDEMA

Interestingly, individuals who have mutations in connexin 47 (CX47) do not appear to develop spontaneous (congenital) lymphedema, but are at significantly increased risk for secondary surgically-associated lymphedema [231]. The significance of this finding may be related to the relatively higher expression of CX47 in lymphatic endothelial cells compared to blood vascular endothelial cells. Therefore, CX47 disturbances could lead to a failure in the appropriate restitution of lymphatic communication after therapy, whereas CX47 might play less of a role in the organization and patterning of lymphatics developmentally. Similarly, lymphatic endothelial cells also express CX37 and CX43.

Because CX37 and CX43 are thought to also participate in the development of lymphatic valves, and CX37 is confined to the external valve leaflet, many believe that these molecules participate in forming valves [69] and also participate in separation of the blood and lymphatic compartments.

## 3.24 ORGAN TRANSPLANTATION AND LYMPHATICS

All forms of solid organ transplantation are known to require reestablishment of the blood vascular supply to maintain transplant survival, however re-anastomosis of lymphatics is rarely considered, again based on the difficulty in visualizing lymphatics and the delicate nature of these conduits. When intestinal tissues are transplanted, lymphatics and blood vessels appear to regenerate independently of each other [232]. Lymphatic expansion often persists following inflammation whereas blood vessels will regress, even so lymphatics must continue to support functions of the blood circulation irrespective of the anatomical location in question. Different influences of lymphatic on transplantation outcomes may depend on structure and function of lymphatics in each organ system.

Although blood vessels must be re-anastomosed to maintain tissue nutrition, lymphatics are rarely reconnected and must "find their own way." Kellersman et al. [233] showed that reconnecting lymphatics following small bowel transplants significantly improves success, an effect which may reflect the large nutritional and immune fluxes supported by these lymphatics. Still, this does not seem to be a requirement, and tissue transfer does not appear to provoke edema as long as the lymph formed may be allowed to passively drain until lymphatic connections can reform. If these channels were obstructed, they would lead to injury. As one example, the

intentional obstruction or blockade of lymphatics in the gastrointestinal system will produce intense tissue injury [234].

By comparison, in liver transplants, lymphostasis (as revealed by CT imaging) may appear as a result of the absence of lymphatic reconstruction in liver transplants. This finding will spontaneously resolve through the gradual regeneration of new lymphatic collaterals [235], even when lymphatic reconstruction is performed.

In kidney transplantation, it has been shown that transplant rejection (renal allograft fibrosis) is associated with reduced blood capillaries (reflecting poor perfusion), but with increased lymphangiogenesis, which appears to intensify injury status. These effects were suppressed by angiotensin converting enzyme inhibitors [236]. We already mentioned a similar role of lymphatics in corneal rejection (see section __ on The Eye).

In the transplanted heart, lymphatic vessels are not reconnected as part of the implantation procedure and lymph formed is returned to the circulation via lymphovenous, epicardial, and pericardial resorption of lymph draining blindly into the pericardial cavity from severed cardiac lymphatics [237]. It is interesting that allogeneic heart transplantation (with only minimal immunosuppression) showed a decreased myocardial lymphatic density—which may be deleterious by failing to clear interstitial fluid. This effect was reversed by an isogeneic (similar) re-transplantation [238] where lymphatic density was higher.

Similarly, in clinical (human) cardiac transplantation studies, when immunosuppression is applied, there is an early decrease in lymphatics [239] and patients with evidence of rejection show increased elevations in VEGFR-$3^{+}$ lymphatics. These results might suggest that more lymphatics are not always better at every stage following transplantation. Sufficient lymphatics may accomplish fluid balance, but too many may lead to immune mediated rejection. We clearly need to study how to strike this balance and suppress inflammatory lymphangiogenesis, which may be beneficial in improving success in the transplanted heart.

## 3.25 EVIDENCE FOR GENES THAT CAUSE ABNORMAL THORACIC DUCT/INTESTINAL LYMPHATIC SYSTEM STRUCTURE

Although several errors in lymphatic master governors like VEGFR-3, FOXC2 and SOX18 are known to disturb lymphatic development and cause lymphedema, such defects can only account for a small number of the cases of congenital lymphedema. Robert Ferrell and colleagues (2008) also reported mutations in families with congenital lymphedema linked to elastin

microfibril interfacer (EMILIN1), lymphocyte cytosolic protein 2 (LCP2), fatty acid binding protein 4 (FABP4), protein tyrosine kinase SYK (SYK), neuropilin-2 (NRP2), SpSRY-box 17 (SOX17), VCAM-1, ROR orphan receptor C, and VEGF-B.

Interestingly, several of these regulators (FABP4, NRP2, SOX17, and VCAM-1) appeared to be a separate group of mutations linked with lymphedema. The endothelial adhesion molecule VCAM-1 appears to be regulated by SOX-18 [37] but this relationship is not understood. However, because EMILIN1 has been described as an α4β1 integrin ligand (like VCAM-1) and is also part of the system which anchors lymphatic endothelial cells to the tissues, if this system is disturbed, it is possible that tension may not be appropriately transmitted to cause lymphatic flap opening, which might lead to lymphostasis. EMILIN2 is also found in large arteries where it may influence immune cell traffic, and it may accomplish the same function in lymphatics as well [240]. EMILIN1 deficient mice show lymphedema, lymphatic overgrowth, and distension, again potentially due to the lack of EMILIN1 dependent tension on lymphatics [241].

Dellinger et al. [242], working on the "Chy-3" (which has only one VEGF-C allele), found that this gene dosing produces chylous ascites, limb, skin and genital edema; the gastrointestinal tract was not affected. The angiopoitetin deficient (Ang-$2^{-/-}$) mouse does not develop normal skin (ear) lymphatics and has disturbed mesenteric lymphatics [73]. These mice fail to develop normal lymphatic collecting vessels and show intestinal lymphangiodysplasia but this is a complex phenotype also involving blood vessel remodeling as well. In mice as in humans, single FOXC2 mutations produce lymphedema distichiasis with both showing disturbances in lymphatic development, especially valve defects and lymph reflux [195]; homozygous FOXC2 deletions in mice are lethal.

## 3.26 RELATIONSHIPS BETWEEN LYMPHATIC MODULATORS AND OBESITY

It has been reported that mice that are genetically deficient for Prox-1 (Prox-$1^{+/-}$) develop lymphedema and have structural abnormalities in their intestinal and mesenteric lymphatics [9]. This hemizygous Prox-1 phenotype shows low perinatal survival; those individuals that survive go on to develop an adult onset form of obesity (but not diabetes). As pups, Prox-$1^{+/-}$ mice have normal insulin and leptin levels, but develop progressive insulin and leptin resistance as adults [10] which results from a loss of lymphatic integrity and lymph leakage which drives subcutaneous and abdominal fat cell (adipocyte) fat accumulation. Therefore, disturbances in

lymphatic integrity (or lymphostasis), which might provoke loss of lymphatic compartmentalization of lipids, might be an important mechanism for obesity development. This is an important change in how we may view obesity, with this study supporting investigations on obesity at least considering maintaining or restoring impaired lymphatic integrity.

Lymphedema exhibits tissue fibrosis and provokes abnormal fat depot stores, and patients with surgically-induced lymphedema who either are obese or gain weight postoperatively are at higher risk for developing lymphedema. Interestingly, increased abundance of adipose tissue often appears to be a prominent feature of both forms primary and secondary forms lymphedema. In this case, debulking by liposuction has been used as treatment to reduce excess tissue volume [243] and may be a long-lasting solution for individuals with lymphedema, even in individuals without normal lymphatic patterning and signaling.

Conversely, the state of obesity itself could also provoke stresses which intensify inflammation and edema and may intensify lymphedema even without a surgically imposed stress [244]. It is noteworthy that body mass indices of 30 and higher show clear evidence of lymphatic transport dysfunction, which appears to be proportionate to the level of obesity (as measured by lymphoscintigraphy) which occurs in patients with no classical risks (congenital or surgical issues).

The presence of obesity causes significant signaling disturbances in many hormonal networks which negatively impinge on health risks. Obesity has been reported to increase both blood and lymphatic vascular regulators including VEGFs C and D, soluble inhibitors of vascular remodeling like sVEGF-R2, as well as activators of angiogenesis such as angiopoietin-2, angiogenin, and endostatin [245]. Changes in the abundance of these factors will also alter fat tissue blood and lymphatic vessel densities. Still, exactly how these obesity-induced vascular regulators affect lymphatic integrity remains unclear, and how different fat "depots" may be affected is not well understood. Serum levels of VEGF-D shows sexual dimorphism with higher levels of VEGF-D present in in lean females than lean males, although the significance of this is not clear. Obese females have higher levels of the proangiogenic/lymphangiogenic factors VEGF-C and Angiopoietin-2 than obese males. Changes in these factors might therefore be controlled by metabolic status and could represent a link between obesity, angiogenesis and inflammation.

## 3.27 TREATMENTS FOR LYMPHEDEMA

Treatments for lymphedema can be symptomatic as well as prophylactic, and should incorporate an effective psychological management approach. This is essential for patients with lymphedema

who may perceive and face perceptions that lymphedema is disfiguring, often an enormous self-esteem challenge in our image focused society. An effective lymphedema management approach will employ massage to promote lymphatic drainage in affected limbs; compressive apparel such as compressive sleeves (especially for arm lymphedema) wraps and elastic bandages.

## 3.28 COMPRESSIVE GARMENTS AND LYMPHEDEMA

Compressive sleeves, garments, and bandages (wrapping) represent an important method for treating individuals with forms of lymphedema. Individuals wearing appropriately fitted arm garments and support stockings may be able to limit the accumulation of interstitial fluid in their extremities and may prevent more longstanding development of chronic lymphedema. Several types of garments come in different levels of compressive force which transmit pressure to the tissue. These devices must be carefully fitted by clinicians and healthcare providers to provide appropriate support for the patient while avoiding impaired blood perfusion. Some of the considerations for the use of these garments include the age of the patient, their level of activity motion, and several other factors including other co-morbidities such as cardiovascular problems, all of which may affect the tissue perfusion and the amount of fluid likely to be generated. Additionally, several important considerations include: avoiding excessive limb overuse; injury; blood donation or the need to take blood samples, and the gravitational and positional effects of letting limbs hang down (which increase the driving force for the production of interstitial fluid).

## 3.29 DRUG TREATMENTS FOR LYMPHEDEMA

Several drug treatments have also been proposed for treating lymphedema including anticoagulants and vascular growth factors as well as "natural" remedies. It has been suggested that experimental treatments intended to expand lymphatic networks might carry some potential risks of support metastatic spread of cancer cells, and drugs aimed at maintaining or improving existing lymphatic function may not be effective.

## 3.30 SURGICAL APPROACHES

One method which has recently been used to prevent surgery-induced lymphedema includes collagen-fibrin patches (Tachosil™), which has been widely employed in obstetric gyneco-

logical reconstruction [247]. A 2014–2017 clinical trial using surgical sealing patch to maintain lymphatic drainage following axillary lymph node dissection in breast cancer (clinical trial NCT02311543) is completed but still under study, and hopefully positive findings will help minimize future surgical complications.

## 3.31 COUMARIN

Coumarin is a benzopyrone anti-coagulant which has been used to treat lymphedema outside of the United States. The mechanism of action of coumarin has been assumed to be to decrease the protein content in the interstitium of the lymphedematous limbs and/or tissues, but this has not been fully supported by clinical trials. Although an open label trial of Coumarin, benzo-gamma-pyrone, and proanthocyanidins relieved transient lymphedema produced by orthopedic surgery [248], coumarin has also been reported to provoke hepatotoxicity. Therefore coumarin use is not currently recommended by the FDA for the treatment of lymphedema.

## 3.32 HORSE CHESTNUT EXTRACT (HCE)

Extracts of horse chestnut (*Aesculus hippocastanum*) contain several principles such as antioxidants flavonol o-glycosides [249], and HCE has been reported to have some benefit in the treatment of chronic venous insufficiency [250], a condition which has some clinical symptomatic and structural similarities to forms of lymphedema. A clinical trial (NCT00213928) on this approach in lymphedema has not yet posted results.

## 3.33 FLAVONOIDS

AdipoDren, a mixed extract of flavonoids, appears to have beneficial effects at least against interleukin-1b mediated lymphatic leakage. The underlying mechanism for this benefit may reflect the stabilization of endothelial tight junctional occludin and ZO-1, as well as suppression of cyclooxygenase (COX)-2. Additionally, this treatment also preserves endothelial and inducible nitric oxide synthases with increased catalase and superoxide dismutase (SOD)-1 and decreased p22phox NADPH oxidase expression [251].

We have also reported that interleukin-1b interferes with lymphatic muscle cell functioning via COX-2 mediated loss of tonic contractility, an effect which is largely reversed by

Anakinra (Kineret) [13], suggesting that this preparation may have benefit in the setting of lymphedema. In a review of botanicals for the treatment of lymphedema, Poage et al. [252] found mixed results for lymphedema, but suggested that it may have some benefits in chronic venous insufficiency.

## 3.34 TACROLIMUS

Because T-cell infiltration of lymphedematous tissues suppresses lymphangiogenesis, the localized (topical) use of Tacrolimus (ProGraf), a T-cell inhibiting transplant immunosuppressant drug, has been tested showing beneficial effects on lymphatic collecting vessel pumping [253]. Such localized use may site-specifically improve lymphatic structure and function while avoiding some undesirable complications of Tacrolimus, which include immunosuppression and neurological problems.

## 3.35 TGF-BETA ANTAGONISTS

Transforming growth factor-beta-1 (TGF-b1) is another growth factor which appears to have a negative impact on lymphatic regeneration and TGF-b may contribute to disturbances in lymphangiogenesis provoking more intense lymphedema. In a model of wound repair, Clavin et al. [102] reported that TGF-b1 disturbed lymphangiogenesis that was producing fibrosis and disturbances in lymphatic remodeling. In that model, collagen used in tissue repair was also seen to influence TGF-b1, suggesting that the use of collagen surgical patches may exert benefits through this pathway. Similarly, TGF-b1 antagonists may also be useful in activating beneficial lymphangiogenic responses to limit or reverse lymphangiogenesis.

## 3.36 TUMOR NECROSIS FACTOR-α (TNF-α) ANTAGONISTS

We have previously demonstrated a negative impact of tumor necrosis factor-alpha (TNF-α) on the proliferation, structure, and function of lymphatic vessels [106]. With respect to therapy, it is striking to note that Polzer et al. [254] found that in arthritis, the clearance of circulating TNF-α achieved (using anti-TNF biologics, which are widely used to treat arthritis and IBD) significantly increased the abundance of lymphatic vessels in the joint tissue. One of the as-

sumptions that might be raised by this study is that the expansion of these more abundant and presumably less-inflamed lymphatics could help to clear inflammatory immune cells and their products from the inflamed joints and speed up resolution of injury. Therefore, in conditions in which normal and beneficial lymphangiogenesis may help to create adaptive lymphangiogenesis, if excess levels of TNF-a are present, lymphangiogenesis may be impaired or vessels rendered less functional leading to edema and chronic inflammation. Consequently, TNF-blockers may be useful in maintaining and or restoring lymph outflow.

Besides anti-TNF-α, thalidomide has also been used as an orally administered small molecule inhibitor of TNF-a messenger RNA [255], which reduces peak TNF-a levels. This might be the case in persistent inflammation (which has been shown for arthritis) and perhaps other forms of inflammation-impaired lymphangiogenesis, however whether and when pharmacological TNF-α blockade might be useful in the setting of secondary forms of lymphedema is unclear (e.g., in IBD).

## 3.37 LEUKOTRIENE $B_4$

One of the most interesting recent findings has been the identification of leukotriene B4 ($LTB_4$) as a therapeutic target in post-surgical lymphedema [256]. Although low levels of $LTB_4$ appear to support lymphangiogenesis, higher levels may actually suppress it. This could reflect the numbers and types of immune cells which are induced to penetrate into tissues and the complement of factors they deliver. Consistent with this concept, the measured high levels of $LTB_4$ in patients with lymphedema could explain at least part of the symptomatology of this condition, with leukotriene synthesis and receptor antagonists being used to therapeutically adjust these levels to restore normal lymphangiogenesis.

## 3.38 THERAPEUTIC LYMPHANGIOGENESIS

The intentional induction of lymphatic vessels by administration of growth factors like VEGF-C and VEGF-D may also be beneficial in the setting of lymphedema, at least acutely. Although we and others found that induction of lymphatic growth factors such as VEGF-D was beneficial in the setting of experimental IBD, this can be highly model specific. For example Gousopoulos et al. 2017 (REF NEEDED) found that administration of VEGF-C, a potent lymphangiogen, also caused blood vessel leakage which increased local edema.

On the other hand, in a model of inflammatory bowel disease, D'Alessio et al. found that VEGF-C was protective against development of injury [257]. By comparison, Wang et al., 2016 found it to be inflammatory [258]. Depending on the particular conditions used in each model, these responses may reflect factors besides VEGFR-3 expression [259] and could include the abundance of endogenous antagonists of lymphangiogenesis such as sVEGFR-2 [260] and sVEGFR-3 [124].

Individuals suffering from diseases in which VEGFR-3 expression and signaling are impaired (such as Nonne-Milroy's disease) might experience less benefit from treatment with such growth factors, although the Milroy's-like lymphedema described by Gordon et al. 2013 (in which a mutant form of VEGF-C is present) might benefit from administration of normal VEGFR-3 ligands [198].

As previously described, because adrenomedullin also enhances lymphatic endothelial ERK1/2 signaling, it may enhance lymphatic proliferation. These effects are reinforced by Prox-1 dependent regulation of adrenomedullin [261] via calcitonin-receptor like receptor (CALCRL), which acts as an adrenomedullin receptor in the presence of RAMP2 [262]. CALCRL itself depends on Prox-1-dependent transcription and adrenomedullin, CALCRL, and RAMP2 knockout mice exhibit lymphedema with perinatal mortality. It is however less clear how restoring these mediators might maintain or restore lymphatics, particularly when other necessary signaling modules may be absent, as in congenital forms of lymphedema.

CHAPTER 4

# Seminal Advancements and Future Directions

The complex structure, function, and rapid remodeling of lymphatics supports the absorptive and immune functions of many different anatomic regions. Because of the critically important roles of the lymphatic system in controlling diverse pathological processes, nature has prepared most tissues with a remarkable ability to increase basal lymphatic flow. Overextended or obstructed lymphatics cannot adequately carry or propel interstitial fluid which will intensify tissue injury and inflammation, both by edema as well as the persistence of immune cells and increased residence time of inflammatory mediators released by infiltrating and resident cells. Chronic edema can oppose tissue perfusion, leading to remodeling, loss of function, and inappropriate immune responses. Tissues are also able to rapidly mobilize and recruit lymphatics to permit homeostatic control of interstitial fluid balance and immune cell recruitment when this ability to accommodate increased lymph flow is overwhelmed.

When hereditary errors in lymphatic structure and function are present, their induced congenital disturbances in lymphatic patterning and development as well as integrity usually produce the many forms of primary lymphedema which we described earlier. This failure to clear lymph from tissues can leave materials in the tissue which should be drained by these conduits, usually water, but also lipids and immune cells can also be confined in tissue with impaired lymphatic egress, leading to a variety of drainage related problems. Thus both the amount of lymph and composition of lymph in addition to the patterning and state of activation of lymphatic networks can lead to many different disturbances and levels of disease severity.

Lymphatic transport is also a "double-edged sword." For example, although failure to clear interstitial fluid produces edema and inflammation, we have also seen how perilous the communication of destructive mediators and infectious pathogens from distant sites of inflammation and infection can be by spreading these life-threatening mediators and organisms which cause remote or systemic organ injury [263]. Such transport by lymphatics can also help spread cancer cells that gain access to the lymphatic compartment and metastasize to distant sites.

Over the past two decades many new and specific structural and gene-controlling biomarkers that can define and modify the activities of the lymphatic system have been identified by several international laboratories. The identification of these genes and proteins not only help us identify when, where, and how lymphatics change during development, normal life, and during disease processes—now we are also learning important ways in which we can modify and enhance the activity of lymphatic expansion, allowing benefits in the control of edema. Similarly it may soon be possible to effectively rebuild lymphatics in tissues in which lymphatics have been destroyed whether through chemotherapy or surgical trauma, which leads to the devastating outcomes following mastectomy and reconstruction in many cases. These molecular therapeutic approaches may also soon rectify congenital disturbances which lead to the stresses associated with inherited forms of lymphedema and lymphedema related disorders.

Conversely, we are now actually able to use suppression of the lymphatic system to limit the lymphatic spread of cancer in many models of conditions, such as in solid tumors like breast cancer, and we may soon be able to "translate" these astonishing findings into the clinic where they are desperately needed. Although we understand the cardiovascular system fairly well at the molecular and cellular level, we are rapidly gaining in a similar understanding of how the lymphatic system participates in cooperation with the cardiovascular system; how it functions as a central component of the immune system, and perhaps even playing important roles in the central nervous system (especially in neurodegenerative diseases). The use of a large variety of animal models (such as global, tissue-specific or inducible) has yielded invaluable information as to when and how lymphatics are functioning well and when they may be contributing to the onset and exacerbation of disease.

Thus, although we now have a greater understanding of the molecular and clinical structure and function of lymphatics in both health and disease, we are only beginning to fully appreciate the full range of their participation in many conditions, both inherited, iatrogenic (originating from treatment), and secondary to cancer or injury. A fuller elucidation of lymphatics, and the mediators which govern and dysregulate their structure and function, is needed. Better methods to image them are also needed, but are advancing rapidly. So far, study in this area shows that we are only beginning to understand this system, and much more basic and clinical research is warranted. As one of the last unexplored anatomic territories, lymphatic biology has tremendous possibilities.

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